UNIVERSIDADE DE LISBOA

Faculdade de Medicina

Mitochondrial Adaptations in Breast Cancer during cell migration: Influence of lipid environments and response to Taxanes

Ana Patrícia Quintino Lopes

Orientadores:

Professor Doutor Sérgio Jerónimo Dias

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Tese especialmente elaborada para a obtenção do grau de Mestre em Oncobiologia

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A impressão desta dissertação foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 15 de janeiro de 2019.

"Os cientistas verdadeiramente grandes são conhecidos pela sua humildade, pois, apesar de explorarem e trazerem à luz os segredos do desconhecido, o desconhecido não cessa de se agigantar e de se tornar cada vez mais misterioso."

Deepak Chopra

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Integrar a equipa de um laboratório significou conhecer uma nova realidade e enfrentar novos desafios que contribuíram para o meu enriquecimento e desenvolvimento pessoal. Depois de um ano de trabalho, o balanço não poderia ser mais positivo.

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Resumo

Durante o desenvolvimento e progressão do tumor, as células cancerígenas adquirem um conjunto de caraterísticas – *the hallmarks of cancer* – que contribuem para o crescimento, proliferação e invasão do tumor. Uma dessas características reside na capacidade das células malignas reprogramarem o seu metabolismo celular. Em 1926, Otto Warburg constatou que, as células tumorais adotavam preferencialmente a glicólise em relação à fosforilação oxidativa como forma de obtenção de energia mesmo na presença de oxigénio, dando particular relevância a esta característica das células tumorais. Este fenómeno, denominado "efeito de Warburg" é também conhecido por glicólise aeróbica e é uma característica transversal à maioria dos tumores. Desde então, múltiplas adaptações metabólicas têm sido descritas, incluindo, por exemplo, a elevada síntese de ácidos gordos e a sobreexpressão de enzimas específicos de determinadas vias metabólicas. Além disso, estudos recentes têm demonstrado que o "efeito de Warburg" não é uma condição *sine qua non* de todos os tumores e a fosforilação oxidativa assume um papel relevante na agressividade do cancro, nomeadamente num contexto de migração e invasão das células tumorais.

Atualmente, o cancro da mama é o tipo de cancro mais frequente e o segundo mais mortífero entre mulheres. Em parte, este problema resulta da enorme complexidade do tumor do ponto de vista molecular e morfológico, o que se traduz numa ampla diversidade de situações clínicas que, por sua vez, são afetadas pelas caraterísticas biológicas do hospedeiro, contribuindo ainda mais para a heterogeneidade tumoral. Existem diversos tipos de cancro da mama, sendo estes classificados de acordo com a presença ou ausência de expressão de recetores hormonais de estrogénio (*ER*) e progesterona (*PR*) e do recetor do fator de crescimento epidérmico 2 (*HER2*). Devido às caraterísticas intrínsecas de maior agressividade e à falta de terapêutica dirigida, o cancro da mama triplo-negativo apresenta pior prognóstico comparativamente aos outros subtipos.

O microambiente do tumor assume um papel crucial na progressão do cancro, uma vez que as células não-malignas que constituem o estroma tumoral produzem e libertam moléculas que podem ter uma função pro-tumoral. A par dos macrófagos e dos fibroblastos, os adipócitos são um dos componentes principais do estroma do cancro da mama e existe um importante *crosstalk* entre estes e as células tumorais, através da libertação recíproca de moléculas sinalizadoras, metabolitos e citocinas, que favorecem o desenvolvimento da doença. Além disso, os adipócitos podem fornecer lípidos às células tumorais que, por sua vez, podem ser utilizados como "combustível" para o crescimento e proliferação do tumor. Adicionalmente, a maioria dos

tumores apresenta desregulação do metabolismo lipídico, fator que potencializa os efeitos anteriormente mencionados, contribuindo para um fenótipo mais agressivo. Os estudos epidemiológicos não oferecem dados muito consensuais acerca da associação entre elevados níveis de LDL e elevado risco de cancro da mama. Contudo, um estudo recente mostrou que determinadas variações genéticas associadas a elevados níveis de colesterol correlacionam-se positivamente com risco de cancro da mama. Adicionalmente, o nosso Laboratório mostrou que, durante o diagnóstico do cancro da mama, mulheres com elevados níveis de LDL desenvolvem tumores maiores e com maior índice proliferativo e, *in vivo,* ratos sujeitos a dieta rica em colesterol também apresentam estas caraterísticas: tumores maiores e com maior índice proliferativo. Observou-se também em estudos *in vitro*, em ambientes ricos em LDL, o aumento da capacidade migratória e proliferativa, bem como a perda de expressão de proteínas envolvidas nos mecanismos de adesão celular (caderina, claudina 7 e ocludina, por exemplo) e a sobreexpressão de intermediários das vias Akt e ERK, ambas responsáveis por uma resposta de sobrevivência. Contudo, os mecanismos moleculares subjacentes à maior agressividade tumoral induzida pela exposição a LDL permanecem pouco esclarecidos.

Além da reprogramação do metabolismo celular, as células tumorais também apresentam frequentemente disfunções da dinâmica mitocondrial. As mitocôndrias são organelos versáteis, capazes de modelar a sua forma e distribuição na célula, consoante as funções que estão a desempenhar e as necessidades da mesma. Relativamente à dinâmica, as mitocôndrias podem apresentar-se com uma forma mais oval e alongada, designando-se por "mitocôndrias elongadas" ou mais redondas e pequenas denominando-se por "mitocôndrias fragmentadas". O balanço entre mitocôndrias elongadas e fragmentadas é controlado por um processo de fissão-fusão, bastante regulado do ponto de vista fisiológico e que se encontra alterado nas células tumorais. Por exemplo, as células tumorais apresentam essencialmente uma rede mitocondrial mais fragmentada e tal está associado a um fenótipo mais agressivo. Em relação à distribuição celular, a rede mitocondrial pode localizar-se preferencialmente em torno do núcleo – rede perinuclear - ou distribuir-se ao longo de toda a célula, sob a forma de longos filamentos – rede filamentosa. Neste processo, o citosqueleto com o auxílio de proteínas motoras desempenham um importante papel na mobilização da rede mitocondrial ao longo da célula. Tendo em conta todos estes aspetos, não é surpreendente que as células tumorais consigam reprogramar o metabolismo, a morfologia, a dinâmica e a própria distribuição da rede mitocondrial na célula, em função do microambiente que as rodeia e do que é mais favorável para o crescimento e proliferação do tumor.

A reprogramação do metabolismo lipídico pode estar associada à disfunção mitocondrial, no entanto os mecanismos envolvidos na regulação destes dois fatores permanecem pouco esclarecidos, nomeadamente no contexto de migração e invasão das células tumorais.

Outro problema responsável pela elevada taxa de recidiva e de morte em pacientes com cancro, é a aquisição de resistência às terapêuticas. Devido à falta de terapêutica dirigida, em pacientes com cancro da mama triplo-negativo este fator encontra-se agravado, uma vez que os pacientes estão sujeitos às terapêuticas mais convencionais, nomeadamente à quimioterapia. Estudos recentes têm reportado a disfunção mitocondrial e a reprogramação do metabolismo lipídico como mecanismos de resistência à quimioterapia em cancro da mama. Deste modo, a caraterização dos aspetos morfológicos e moleculares subjacentes à reprogramação metabólica e à disfunção mitocondrial envolvidos na resistência à quimioterapia são importantes, pois podem contribuir para o desenvolvimento de eventuais terapias dirigidas e desse modo reverter a resistência aquirida.

O presente estudo focou-se na resposta a duas questões principais: por um lado, pretendeu-se compreender de que modo as mitocôndrias se adaptam à exposição a LDL e como é que isso de algum modo contribui para um aumento da capacidade migratória em cancro da mama triplo-negativo; por outro lado, também decidiu-se explorar a influência do papel da exposição crónica a um agente quimioterapêutico, nomeadamente o Taxol, no fenótipo de células tumorais de cancro da mama triplo-negativo (mais especificamente, na capacidade migratória e proliferativa e na massa mitocondrial), bem como se a exposição a LDL também influencia estes parâmetros nestas mesmas células. No geral, os nossos resultados sugerem que o fenótipo agressivo provocado pela exposição a LDL, em células de cancro de mama triplo-negativo, é acompanhado por alterações em diversos parâmetros relacionados com a morfologia e rede mitocondrial. Adicionalmente, os nossos resultados mostram que este facto também poderá ser dependente de interações específicas entre mitocôndria e citosqueleto. Por sua vez, no contexto de resistência à quimioterapia, os dados mostraram que os efeitos produzidos pelo LDL são particularmente pronunciados após uma exposição prolongada ao Taxol e não durante o tratamento. Assim, este estudo fornece novos conceitos acerca do papel do LDL na agressividade do cancro da mama triplo-negativo, incluindo no contexto de resistência à terapia, um assunto ainda muito pouco explorado, providenciando, deste modo, potenciais ideias que poderão ser relevantes para realização de estudos posteriores e as quais poderão ter interesse do ponto de vista clínico.

Palavras-chave: Metabolismo Tumoral, Cancro da Mama Triplo-Negativo, Mitocôndria, LDL, Resistência Quimioterapêutica

Abstract

Breast cancer remains the most common cancer and the second cause of cancer related death among women despite the significant advances in diagnostic and therapy. In one hand, tumors display great molecular and morphological heterogeneity; on the other hand, host biological characteristics contribute to the wide diversity of clinical situations. During cancer progression, tumor cells acquire specific features, designated as the hallmarks of cancer that enable tumor growth, proliferation and dissemination, which globally may contribute to escape apoptosis and therapeutic intervention. Deregulated cellular energetics is nowadays considered an emerging hallmark of cancer. In particular, abnormal lipid metabolism has been reported as one of the metabolic adaptations exhibited by tumors, including breast cancer, which allows the acquisition of aggressiveness and favors tumor invasion and metastasis. Besides presenting deregulated cellular energetics, tumor cells often exhibit mitochondrial dysfunction. Reprogramming of lipid metabolism has been associated with mitochondrial dysfunction, but the exact nature of the mechanisms underlying this regulation remains poorly understood. Resistance to therapy, including chemotherapy, is a major cause of cancer related death. More recently, it was described that reprogramming lipid metabolism, as well as mitochondrial dysfunction are both involved in the acquisition of resistance to conventional cancer therapy.

In this study, we aim to unravel how mitochondria adapt in high hypercholesterolemic environments (LDL) and if mitochondrial adaptations are required for the acquisition of increased migratory and proliferative phenotype of triple-negative breast cancer (TNBC) under these conditions. Also, we aim at understanding how chronic exposure to a chemotherapeutic agent, Taxol, influences TNBC cells mitochondrial mass and phenotype (migration and proliferation capacity) and if LDL exposure could modulate these parameters. Our results suggest that LDL-induced aggressiveness is accompanied by adaptations in mitochondrial morphology and network arrangement, which could be dependent on specific mitochondria-cytoskeleton interactions. Regarding response to chemotherapy, our data show that the effects produced by LDL exposure are particularly pronounced after prolonged exposure to Taxol and not during acute treatment. Overall, this study provides novel mechanistic concepts about the impact of LDL exposure in the acquisition of TNBC cells aggressive phenotype, including response to chemotherapy, which offer important insights for the development of future studies.

Keywords: Cancer Metabolism, Triple-Negative Breast Cancer, Mitochondria, LDL, Resistance to Chemotherapy.

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List of Abbreviations

ACC1 – Acetyl-CoA carboxilase

ACLY – ATP citrate lyase

ATP – Adenosine triphosphate

BC – Breast cancer

BCSCs – Breast cancer stem cells

BSA – Bovine serum albumin

***B2MG* –** β2-microglobulin gene

CAA – Cancer-associated adipocytes

CPT1B - Carnitine palmitoyltransferase 1B

CSCs – Cancer stem cells

DFS – Disease free survival

DMEM - Dulbecco's Modified Eagle Medium

Drp1 – Dynamin related protein 1

EMT – Epithelial-to-mesenchymal transition

ER – Estrogen receptor

FAs – Fatty acids

FAO – Fatty acid oxidation

FASN – Fatty acid synthase

FBS – Fetal bovine serum

FBS-LPF – Fetal bovine serum lipoprotein free

FDA – Food and Drug Administration

GLS1 – Glutaminase 1

GTPase – Guanosine triphosphate hydrolase

HCC – Hepatocellular carcinoma

HER2 – Human epidermal growth factor receptor 2

HIF1α – Hypoxia-inducible factor 1α

HMG-CoA - 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase

HSP60 – Heat shock protein 60

LDH-A – Lactate dehydrogenase A

LDL – Low density lipoprotein

LDLR – Low density lipoprotein receptor

LDs – Lipid droplets

LPA – Lysophosphatidic acid

LSM – Laser scanning microscope

MBC – Metastatic breast cancer

MCL1 – Myeoloid cell leukemia-1

MDA-MB-231 CE-Taxol – MDA-MB-231 Chronically Exposed to Taxol 2,5 nM

MDA-MB-468 CE-Taxol – MDA-MB-468 Chronically Exposed to Taxol 2,5 nM

MDR-1 – Multi-drug resistance gene

Mfn1/2 –Mitofusin1/2

MRD – Minimal residual disease

mtDNA – Mitochondrial DNA

ND1 – NADH dehydrogenase 1

OXPHOS – Oxidative phosphorylation

PA – Palmitic acid

PDAC – Pancreatic ductal adenocarcinoma

PFA – Para-formaldehyde

P-gp – P-glycoprotein

PR – Progesterone receptor

qPCR – Quantitative polymerase chain reaction

ROS – Reactive oxygen species

RT – Room temperature

TAG – Triacylglycerol

TCA cycle – Tricarboxylic acid cycle

TNBC – Triple-Negative Breast Cancer

1. Introduction

1.1. Breast cancer

Breast cancer remains the most common cancer and represents the second most frequent cause of cancer-related deaths among women. In part, this problem is related to its great heterogeneity and complexity at molecular and morphological levels, which translate into a diverse clinical entity and is affected by its host biological characteristics [1].

Breast cancer can be classified according to the expression and/or lack of expression of hormone receptors, such as estrogen receptor (ER) and progesterone receptor (PR), as well as expression and/or lack of expression of the human epidermal growth factor receptor 2 (HER2). Taking into account its gene expression and immunohistochemistry profile, breast cancer can be classified in luminal A (ER+, PR+/-, HER2-), luminal B (ER+, PR+/-, HER2+), HER2 (ER-, PR-, HER+) and triple-negative (ER-, PR-. HER2-). Among TNBC subtype, it can be further classified into basal-like or claudin-low and the latter one represents an even more aggressive subtype with worse response to chemotherapy [2].

Each subtype of breast cancer is associated with a different prognosis and follows different therapeutic guidelines. Due to its intrinsic aggressiveness, as well as the lack of targeted therapies, TNBC has a poor prognosis when compared to other subtypes of breast cancer [3].

In order to identify and develop potential candidates for targeted therapy, it is imperative to better understand the molecular and morphological mechanisms underlying TNBC aggressiveness, as well as the impact of the tumor microenvironment on this subtype of breast cancer.

1.2. Reprogramming cell metabolism in cancer

In 1926, Otto Warburg first described that cancer cells preferentially adopt glycolysis over OXPHOS (oxidative phosphorylation), even in the presence of oxygen [4]. In 2011, Hanahan and Weinberg proposed a new generation of hallmarks of cancer where deregulated cellular energetics emerged as a new hallmark [5]. Nowadays the so-called “Warburg Effect” is considered a well established feature of most cancers and additionally several other metabolic adaptations that support tumor progression have been described, including increased synthesis of proteins, nucleotides and fatty acids [6,7]. Moreover, recent studies have shown that OXPHOS and mitochondrial respiration play a key role in tumors, especially in tumor migration, invasion and therapy-resistance [8–10].

Among other aspects, tumors rely on these metabolic adaptations in order to sustain cell growth, proliferation and invasion, escaping apoptosis and becoming resistant to conventional therapies, such as chemotherapy.

1.2.1. Reprogramming lipid metabolism in breast cancer

Lipids constitute one of the essentials biomolecules to life, performing structural and bioenergetic functions in the cell. They include fatty acids (FAs), triacylglycerols (TAGs), sterols (like cholesterol), among others [11]. The metabolic pathways responsible for lipids biosynthesis and degradation are very complex and they require the intervention of several metabolites and intermediates, such as signaling molecules, growth factors and chemokines [12–14]. A wide variety of studies report increased expression and activity of lipogenic enzymes in tumors, namely Acetyl-CoA carboxilase (ACC) and fatty acid synthase (FASN), as well as ATP citrate lyase (ACLY) that promotes choleterol synthesis [12,15,16].

Most tumors, including breast cancer, present adaptations and abnormal signaling in pathways linked to lipid metabolism. Tumors take advantage of abnormal lipid metabolism in order to promote their growth and proliferation, allowing the acquisition of a more aggressive phenotype that favor invasion and metastasis [6,11,14,17].

Cancer cells have the ability to store excessive lipids in lipids droplets (LDs), which are small organelles derived from the endoplasmatic reticulum containing TAGs and cholesterol which are surrounded by a phospholipid monolayer [18]. The high amount of LDs in cancer cells has been associated with a more aggressive phenotype. For instance, MDA-MB-231 and MDA-MB-436, two human TNBC cell lines, have more LDs when compared to MCF-7, a human luminal A breast cancer cell line. The same study showed that in the presence of lipid depleted medium, MDA-MB-231 cells exhibit a decrease of LDs content concomitant with a decrease in its migratory capacity [19].

In addition to the energy supply function, lipids also perform structural functions. Beloribi-Djefalia *et al*., reviewed among other aspects the importance of lipid rafts in cancer. Lipid rafts not only control cell membrane dynamics and transport, but also have a key role in cell signaling. In early, advanced and metastatic breast cancer, lipid rafts have the ability to regulate pro-oncogenic and apoptotic pathways and can interfere with cytoskeleton and focal adhesion dynamics, therefore contributing to cancer cell migration and invasion [14].

Taking into account all these aspects, there is strong evidence that targeting abnormal lipid metabolism in cancer could be a promising and innovative strategy to stop cancer cells proliferation and dissemination.

In 2016, Camarda *et al*., showed through metabolomic approach that several intermediates of fatty acid oxidation (FAO) are upregulated in a genetic-driven model of TNBC overexpressing the oncogenic transcription factor MYC (MO-TNBC). The pharmacologic inhibition of FAO with etomoxir led to a decrease of metabolic energy of MO-TNBC and attenuated tumor growth *in vivo* [20]. Moreover, other studies have reinforced the importance of mitochondrial fatty acid β-oxidation as an important energy pathway in metastatic TNBC and the effect of oleic acid in promoting breast cancer cells proliferation through CD36 (fatty acid translocase) [21].

Tumor microenvironment plays a critical role in cancer progression since non-malignant cells that constitute the tumor stroma can produce and release pro-tumoral molecules. Such as macrophages, fibroblasts and endothelial cells, adipocytes are one of the major components of breast cancer stroma. There is a strong crosstalk between adipocytes and breast cancer cells through the exchange of signaling molecules and metabolites. In one hand, breast cancer cells release cytokines and lypophilic enzymes that mediate the establishment of cancer-associated adipocytes (CAAs) with particular features, including delipidation, dedifferentiation and abnormal secretion. On the other hand, adipocytes release, cytokynes, adipokynes and hormones, among other factors, that contribute to a more aggressive phenotype of breast cancer cells. Also, adipocytes can provide lipids that are used as fuels to tumor growth and proliferation [16]. Together, these evidences show the key role of lipid metabolism for cancer progression.

Cholesterol is an essential lipid component in mammalian cellular membranes as it assumes crucial functions for life, such as regulating fluidity and being an important component of lipid rafts [11]. Mammalian cells acquire cholesterol from two different sources: in one hand, cells can uptake cholesterol from extracellular lipoproteins (exogenous source) to the endoplasmatic reticulum (ER), in a process that could be mediated by Aster, as reviewed in a recent study[22]; on the other hand, cells can acquire cholesterol from *de novo* synthesis from glucose (endogenous synthesis) [23]. Breast cancer cells can take advantage on this endogenous synthesis and upregulate glycolysis, as well as FASN and HMG-CoA, both enzymes responsible for fatty acids and cholesterol synthesis, respectively [19]. LDL, also known as low-density lipoprotein, is constituted by multiple proteins and variable amounts of several lipids (including cholesterol, phospholipids and triglycerides) which is responsible for transporting cholesterol from the liver to the tissues [11] (**Figure 1**).

Concerning high levels of LDL and increased risk for breast cancer, epidemiological studies are not very consensual [24–26]. However, it was shown that patients with increased levels of LDL at the diagnosis stage, develop larger and more proliferative breast tumors [27].

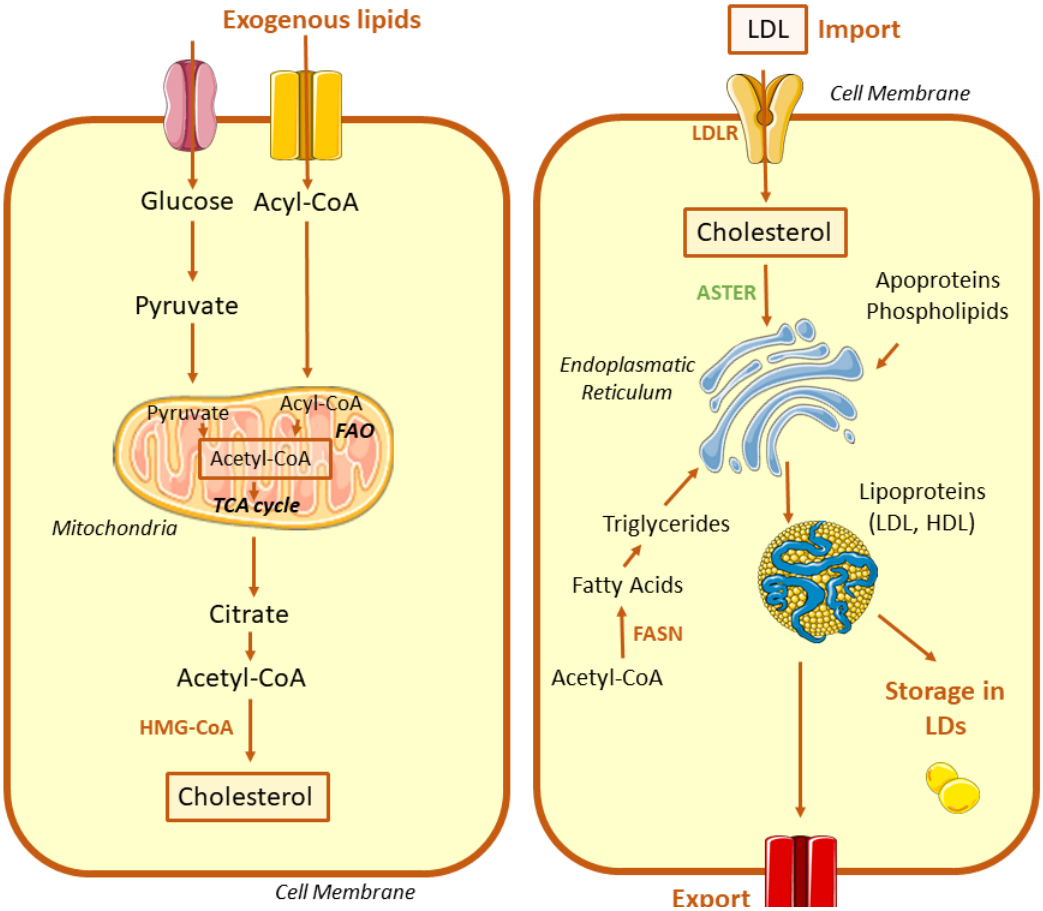


Figure 1. Simplified overview of lipid metabolism.

Mammalian cells acquire cholesterol from two different sources: uptake from extracellular lipoproteins (LDL) – exogenous source - or *de novo* synthesis from glucose – endogenous source. Additionally, cholesterol can be exported to the endothelium in the form of lipoproteins (HDL, LDL) or can be stored in lipid droplets (LDs). Cancer cells, including breast cancer cells, often exhibit deregulated lipid metabolism and present upregulated glycolysis as well as upregulated FASN, enzyme responsible for fatty acids synthesis.

Moreover, a recent study involving more than 400.000 patients showed that genetically raised LDL-cholesterol is associated with higher risk for breast cancer [28].

Cancer cells additionally present increased uptake of exogenous circulating lipids, such as LDL. Recently, our lab showed that LDL plays an important role in modulating breast cancer progression using both *in vitro* and *in vivo* cancer models. LDL exposure promotes epithelial-to-mesenchymal-transition (EMT) features, increased proliferation and migratory capacity, as well as loss of adhesion with decreased expression of adhesion molecules such as cadherin-related family member3, claudin 7 and ocludin. Moreover, mice fed with a high-cholesterol diet present larger and more proliferative breast tumors and exhibit more often lung metastases,

corroborating with the *in vitro* data [29]. Also, in the clinic, plasma level of LDL can be used as a biomarker for breast cancer progression. Breast cancer patients with elevated levels of LDL at diagnosis, present larger tumors with higher proliferative rate. Additionally, higher levels of LDL are associated with poor disease free survival (DFS) [27]. Despite this knowledge, the exact mechanisms that mediate LDL-induced aggressiveness in breast cancer progression remain unclear.

Taken together, the presented evidence suggests that targeting lipids, and in particular LDL, could be a novel and innovative strategy to prevent breast cancer growth and dissemination. A review from Beloribi-Djefalia *et al*., show a set of ongoing studies, mostly preclinical trials, that explore the use of lipogenic enzyme inhibitors as anticancer therapy, including statins and etomoxir; reinforcing the emerging importance of lipid metabolism as potential targets for anticancer therapy [14].

1.2.2. Mitochondrial dysfunction in breast cancer

Mitochondria are dynamic organelles, well known to be the "powerhouse of the cell" since they provide most cellular energy, either by generating ATP through OXPHOS or by contributing to the synthesis of several metabolites, such as fatty acids and carbohydrates [30]. In addition to its bioenergetic functions, mitochondria also play an important role in controlling intracellular calcium concentration and in apoptosis [31,32].

Mitochondria perform multiple functions in the cell, so it is essential that they model their shape and size, as well as cellular distribution (**Figure 2**), in order to efficiently meet the cellular demands. Regarding mitochondrial dynamics, mitochondria can exhibit tubular and elongated networks – elongated mitochondria – or they can appear as fragmented, rounded and punctuated structures – fragmented mitochondria. The balance between elongated and fragmented mitochondria is controlled by a fission/fusion process, which in turn is regulated by dynamin related GTPases, in a highly conserved process. After undergoing post-translational modification, Drp1 is recruited by mitochondria, promoting mitochondrial fragmentation and mediating the fission process, whereas Mfn1 and Mfn2 are responsible for mediating the fusion process of the mitochondrial outer membrane [33].

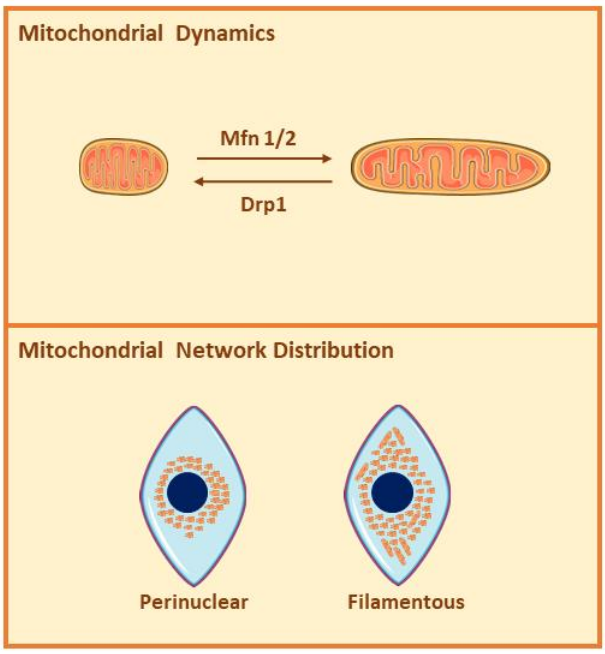


Figure 2. Schematic (simplified) representation of mitochondrial dynamics and mitochondrial network distribution.

Mitochondria modulate their shape and size through a stricktly regulated fusion/fission process. Fusion induces more elongated mitochondria and it is mediated by Mfn 1/2, whereas fission increases mitochondria fragmentation and it is mediated by Drp1. Mitochondria can also exhibit different subcellular distribution, according to specific cell needs, could appearing more perinuclear or more filamentous arranged throughout the whole cell.

Mitochondria are also able to model their cellular distribution, depending on the cell type and energy demands. They may lie more around the nucleus - perinuclear mitochondrial network – or distribute throughout the cell in long filaments – filamentous mitochondrial network [34]. The cellular distribution of mitochondria in the cell requires an interaction with the cytoskeleton, including microtubules or actin filaments. This interaction also requires the participation of motor proteins, such as myosin that assists movement through actin filaments and dinein and kinesin, which help in the movement through the microtubules[35].

Mitochondria play a key role in cancer and its dysfunction is present in breast cancer, providing a wide diversity of advantages to the tumor cell. Cancer cells are able to reprogram their cellular metabolism in order to sustain their proliferation and growth and they can also regulate

mitochondrial dynamics according to what favors tumor the most. Additionally, mitochondria may also take advantage of the interaction with cytoskeleton and be mobilized to certain regions of the cell that facilitates invasion and migration processes.

In cancer, the interaction between mitochondria and the cytoskeleton has an important role. As motor proteins regulate and drive mitochondria distribution to sites of high-energy demand, this can provide an advantage to tumor cells in certain processes, such as migration and invasion. Indeed, in MDA-MB-231 cells, the accumulation of mitochondria in the leading edge is concomitant with a higher velocity of migration [36].

Mitochondrial dysfunction is associated with deregulated cellular energetics and this includes not only adaptations of mitochondrial metabolism, but also changes in mitochondrial dynamics, cellular distribution and mitochondrial DNA content [30,33,37]. For instance, mitochondria are more fragmented in metastatic breast cancer and Drp1 expression is increased in human invasive breast carcinomas [31]. Also, circulating tumor cells exhibit a higher mitochondrial DNA content when compared to primary cancer cells, suggesting that cancer cells can remodel mitochondria mass and number in a way that most efficiently favors its progression within a particular environment [9]. Recently, a study described a molecular mechanism in which ACC1, a lipogenic enzyme involved in the regulation of *de novo* FA synthesis, contributes to the increase of mitochondrial fission in pluripotent stem cells [38], providing a mechanistic link between lipid metabolism and regulation of mitochondrial dynamics.

Together, these facts suggest that understanding the molecular mechanisms underlying mitochondrial network distribution and its specific interaction with lipidic metabolism and the cytoskeleton could be a promising approach to find new potential targets for anticancer therapy.

1.3. Chemotherapy resistance in breast cancer

Depending on the breast cancer subtype and the stage of disease progression (early, advanced or metastatic), there is a wide variety of therapies available for treatment. Chemotherapy is used as first line therapy for metastatic breast cancer (MBC) or for patients with TNBC. It is used as adjuvant therapy for patients with MBC that already received local treatment (like surgery, for instance) and have a high risk of relapse. The most common chemotherapeutic agents used in breast cancer treatment include antracyclines, taxanes, gemcitabine, vinorelbine and carboplatin [1,3,39,40].

Despite significantly improvement in anti-cancer therapy, one of the leading causes of cancer-related death in breast cancer is the acquisition of therapy resistance, including to chemotherapy. The lack of targeted therapy for TNBC reduces the therapeutic options for breast

cancer subtype, increasing tumor recurrence associated with resistance to therapy, with development of metastases, cancer spread and worse prognosis [3].

Taxol, also known as paclitaxel, is a microtubule-stabilizing drug belonging to the Taxanes family, widely used as chemotherapeutic agent approved (by FDA) for the treatment of ovarian and breast cancer and, in particular it is used as first line therapy for the treatment of MBC [39,41,42].

Microtubules consist of long protein polymers composed of repeating subunits of α- and β-tubulin which are responsible for important cellular functions, such as cell division, motility, cell shape and signaling [40]. The mechanism of Taxol action lies in the ability to interact with microtubules and interfere with its dynamics of polymerization and depolymerization events, which is essential to cell division and chromosome segregation during mitosis. Taxol binds to Taxol-binding site located in the luminal side of β-tubulin subunit and induces mitotic arrest. Consequently, the metaphase-anaphase transition is inhibited, mitosis is blocked and most cells enter in apoptosis. In some cases, cells can exit mitosis through mitotic slippage, without needing anaphase or cytokinesis. This phenomenon is characterized by producing tetraploid and multinucleated G1 cells that did not undergo chromosomal segregation [40,41,43]. Due to its ability to interfere with microtubules dynamics, Taxol is also used commonly used in biomedical research as a cytoskeleton-destabilizing agent [44–46] .

As for other agents, treatment with Taxol eventually leads to the acquisition of resistance by the tumor cells. McGrogan *et al*., reviewed some mechanisms associated with acquired resistance to Taxol which include mutations in α- and β- subunits that weaken the binding with Taxol and overexpression of P-glycoprotein (P-gp), responsible for the efflux of several drugs leading to decreased intracellular levels and drug resistance [40].

Thus, it is essential to understand the diversity of molecular mechanisms underlying acquired resistance to chemotherapy in order to develop strategies that counteract this phenomenon and re-sensitize cancer cells to therapy.

1.3.1. Metabolic adaptations in chemotherapy resistant (breast) cancer

Lately, reprogramming cell metabolism in tumor cells has been pointed out as one of the features which tumor cells commonly adapt in order to acquire resistance to chemotherapy [47].

The acquired resistance to anti-cancer therapies, including chemotherapy, is often associated to a special population of cancer cells that survives to therapeutic intervention. This cell population is known in the clinics as minimal residual disease (MRD). Recently, a study in mice mimicking MRD reveal that residual breast cancer cells have a defined metabolic signature and a transcriptome distinct from the normal epithelial cells and the primary breast tumor. Also, this study revealed that the population of cells with acquired resistance to therapeutic

intervention present altered lipid metabolism and higher production of reactive oxygen species (ROS) [13].

Additional studies have reported that reprogramming of lipid metabolism is one of the causes of resistance to chemotherapy. For instance, JAK/STAT3 pathway induces fatty acids β-oxidation (FAO) through the transcription of CPT1B, a crucial enzyme of FAO, which in turn promotes breast cancer stem cells (BCSCs) self-renewal and chemotherapy resistance. Additionally, leptin derived from mammary-adipocytes upregulates JAK/STAT3 pathway, further enhancing this effect. Through the inhibition of FAO or leptin, it is possible to inhibit BCSCs and reverse the chemo resistant phenotype [48].

High amounts of LDs are also associated with increase resistance to chemotherapy. It was shown that pancreatic ductal adenocarcinoma (PDAC) presents increased amounts of cholesterol, as well as overexpression of LDL receptor (LDLR) and inhibition of cholesterol uptake resensitized cells to chemotherapy, contributing to PDAC regression [49] .

Mitochondrial dysfunction is also associated with increased resistance to chemotherapy. *MYC* and MCL1, a proto-oncogene and a pro-apoptotic protein of Bcl-2 family respectively, are co-amplified in chemotherapy resistant TNBC. Together, they contribute to increase OXPHOS and ROS levels, both processes involved in the maintenance of CSCs. In turn, the higher levels of OXPHOS contribute to accumulation of HIF1α and blocking HIF1α was shown to decrease CSCs enrichment, decreasing resistance to chemotherapy and attenuating tumor growth *in vivo* [10]

Specifically regarding Taxol, several studies have suggested that targeting the metabolism of tumor cells is a strategy to reverse chemotherapy resistance in different tumor types [50]. In breast cancer, human MDA-MB-435 cells with high LDH-A expression can be resensitized to Taxol either through the use of siRNA that downregulates LDH-A expression or using oxamate, a LDH-A inhibitor [51]. A similar study, showed increased expression of glutaminase 1 (GLS1) in Taxol-resistant MDA-MB-231 cells. Downregulation of GLS1 using siRNA resensitized MDA-MB-231 cells to Taxol whereas overexpression of GLS1 in BT-474, a Taxol-sensitive BC cell line, rendered the cells resistant [52].

In summary, abnormal cell metabolism and mitochondrial dysfunction play a key role in chemotherapy resistance, a major cause of breast cancer-related death. Therefore, it is crucial to characterize the resistant phenotypes and to discover new molecular mechanisms involved in acquired resistance to chemotherapy with the aim of developing alternative strategies to counteract chemotherapy resistance in breast cancer.

2. Aims

TNBC cells are able to reprogram their cellular metabolism in order to sustain tumor growth and progression. Recent data from our laboratory showed that exposure to LDL-enriched environments is accompanied by alterations in mitochondrial mass and dynamics, which could be at the basis of the increased migratory and proliferative phenotype displayed by LDL-exposed cells. Despite this knowledge, the exact molecular implications of the altered mitochondria morphology/network for LDL-induced TNBC aggressiveness remained unclear. Additionally, dysfunction in lipid metabolism and in mitochondrial metabolism and morphology have been reported as one of the main causes of chemotherapy resistance.

Therefore, the aim of this thesis is to study the following two main hypothesis:

- How mitochondria adapt to LDL exposure and how this contributes for the aggressive migratory phenotype in TNBC? Is this process mediated by the cytoskeleton?
- How chronic exposure to the chemotherapeutic agent Taxol influences the phenotypic properties of TNBC cells (namely, migratory and proliferative capacities) and additionally, the mitochondria mass? Moreover, does LDL exposure influence all these features in Taxol acute or chronically-exposed TNBC cells?

Therefore, the specific aims of this study/thesis are:

- **Aim 1.** Characterization of the mitochondrial morphology in LDL-exposed migrating MDA-MB-231 cells.
- **Aim 2.** Determination of the role of cytoskeleton in mitochondrial morphology in LDL-exposed migrating MDA-MB-231 cells.
- **Aim 3.** Establishment of Taxol-resistant TNBC cell lines (MDA-MB-231 and/or MDA-MB-468) *in vitro.*
- **Aim 4.** Determination of the role of LDL in Taxol-resistant TNBC cell lines.

3. Materials and Methods

3.1. Cell Culture

MDA-MB-231, MDA-MB-468, BT-474 and MCF-7 human breast cancer cell lines were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% (v/v) heat-inactivated FBS (Gibco Invitrogen) and 1% Antibiotic-Antimycotic (Gibco Invitrogen), named complete DMEM and incubated at 37ºC and 5% CO_2 atmosphere.

3.1.1. Generation of Taxol-resistant breast cancer cell lines

MDA-MB-231 and MDA-MB-468 cells were cultured in flat bottom 6-wells plates, in complete DMEM and permanently treated with Taxol (Sigma) at a final concentration of 2,5 nM, during 3 months. After this period, it was performed a viability assay to evaluate the sensitivity to Taxol between the parental and chronically exposed (CE) cell lines. For the subsequent experiments, the MDA-MB-231 CE-Taxol cell line was treated with Taxol 2,5 nM to maintain Taxol resistance.

3.1.2. Mito-YFP transfection

MDA-MB-231 cells were seeded at a density of $2x10^5$ cells/mL in 35 mm glass bottom dishes and after 24 hours transfected with 2 µg Mito-YFP using FuGENE (ThermoFisher Scientific) and according to the manufacturer instructions.

3.2. Viability Assays

To test the sensitivity of cancer cells lines to Taxol, cells were seeded at a density of 1 x 10^5 cells/mL in a flat-bottom 24 well-plate and subjected to different Taxol concentrations (0; 1; 2,5; 5; 7,5 and 10 nM). After 5 days, the number of viable cells was determined using Trypan Blue exclusion test by hemocytometer counts (4 squares per well), in quadruplicates. To test the effect of LDL in the response to Taxol, cancer cells lines were seeded at a density of 4 x 10^4 cells/mL in a flat bottom 24 well-plate, in complete DMEM. After 24h, the medium was replaced by DMEM 1% FBS-LPF for 24h. After this period, medium was replaced by fresh DMEM with 1% FBS-LPF only or supplemented with LDL 100 µg/mL (Merck) or Taxol 2,5 nM alone or in combination for 48h. The number of viable cells was determined using Trypan Blue exclusion test by hemocytometer counts (4 squares per well), in quadruplicates.

3.3. Proliferation Assays

Cancer cell lines were seeded at a density of 4 x 10^4 cells/mL in a flat bottom 24 well-plate, in complete DMEM. After 24h, the medium was replaced by DMEM supplemented with 1% (v/v)

FBS-LPF (fetal bovine serum – lipoprotein free) (Bio West) for 24h. After this period, medium was replaced by fresh DMEM with 1% FBS-LPF only or supplemented with LDL 100 µg/mL (Merck) or Taxol 2,5 nM alone or in combination for 48h. The number of viable cells was determined using Trypan Blue exclusion test by hemocytometer counts (4 squares per well), in quadruplicates.

3.4. Wound-Healing Assay

MDA-MB-231 cells were seeded at a density of 1,5 x 10^5 cells/mL in a flat bottom 24-well plates in 500 µL complete DMEM. After 48h, when cells reached confluence, the medium was replaced by DMEM supplemented with 1% (v/v) FBS-LPF for 24h. Blue tips (1000 µL) were used to make a scratch ("wound") in the center of the wells. Cells were washed with PBS 1x and the medium was replaced by fresh DMEM with 1% FBS-LPF, supplemented with Mitomycin C (Merck Milipore; 0,5 µM) only or supplemented with LDL 100 µg/mL or Taxol (20 nM or 2,5 nM) or LPA (Sigma-Aldrich, 10 µM) alone or in combination. Cells were allowed to migrate during 16h (assays with LDL and LPA or with LDL,Taxol 2,5 nM, LDL+Taxol 2,5 nM) or 24h (assays with LDL, Taxol 20 nM, LDL + Taxol 20 nM) and at least two photos per well were taken at starting and ending time in a Zeiss Primovert microscope (4x objective) coupled with a Zeiss AxioCam ERc 5s camara. Cell migration distance was measured by subtracting the wound length at the final time from the initial time, using Fiji software (10 measurements per well in triplicates).

3.5. mtDNA determination

For mtDNA determination, total DNA was isolated from cells using phenol:chloroform:isoamyl alcohol (Sigma) and measured by assessing the levels of the human mitochondrial *NADH dehydrogenase 1* (*ND1)* relative to nuclear *β2-microglobulin* (*B2MG*) gene. qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad), following manufacturer procedures, in an Applied Biosystems ViiA 7 system.

Primer sequences are described in **Table 1.** Quantifications were made applying the ΔCt method (Ct of nuclear DNA gene – Ct of mitochondrial DNA gene), followed 2x2^(ΔCt) according to Rooney *et al*.. [53].

Table 1. Amplified genes and primers used for mtDNA content quantification using qPCR.

Gene name	Genome	Primer	Sequence
B2MG	Nuclear	Forward Seq 5′-3′	TCGCTCCGTGGCCTTAGCTGT
		Reverse Seq 5′-3′	CTTTGGAGTACGCTGGATAGCCTCC
ND1	mitochondrial	Forward Seq 5′-3′	CCCTAAAACCCGCCACATCT
		Reverse Seq 5′-3′	GAGCGATGGTGAGAGCTAAGGT

3.6. Flow Cytometry

For MitoTracker Deep Red (Molecular Probes) staining, cancer cell lines were seeded at a density of 3 x 10^5 cells/mL in flat-bottom 6 well- plates. When cells reached confluence, cells were trypsinized, centrifuged at 1000 rpm , washed with PBS 1x and MitoTracker Deep Red (2 nM) was added in PBS 1x, during 15 minutes – RT. Cells were washed and ressuspended in PBS 1x. For cell death detection, cells were washed in PBS 1x and resuspended in 300 µL of Annexin V-binding buffer (BD Biosciences) containing 5 mL of 7-aminoactinomycin (7-AAD) (BD Pharmingen) and 5 µL of Annexin V-PE (BD Biosciences), and incubated for 15 minutes at RT. Flow cytometry analysis was performed in in a LSR Fortessa (BD Biosciences) flow cytometer followed by data analysis using the Flow Jo Software.

3.7. Immunofluorescence

Cells were seeded in glass coverslips and after culturing, cells were fixed with 4% PFA – 10 minutes, following by permeabilization with 0,5% Triton X-100 – 5 minutes and blocking with 0,5% BSA – PBS – 30 minutes. Cells were immunostained with primary antibody (rat anti-human α-tubulin YL 1/2, at 1:100; mouse anti-human HSP60, BD Biosciences; at 1:250) overnight. On the next day, cells were washed with PBS 1x and incubated with the secondary antibody (goat anti-rat Alexa 594, Invitrogen, at 1:500; donkey anti-mouse Alexa 594, Invitrogen; at 1:500) and/or Alexa Fluor Phalloidin 647 (ThermoFisher Scientific, 1:200) for 1h, at RT. Mounting was performed using Vectashield with DAPI mounting medium.

Epifluorescence microscopy was performed in a Zeiss Axiovert 200M microscope and the images were acquired with a 63x oil objective. Confocal microscopy was performed in a Zeiss LSM 880 microscope and the images were acquired with a 63x oil objective (with 1.6x zoom). The images were processed using Fiji software. Mitochondrial morphological parameters - mean number of mitochondria, mean size of mitochondria, mean interconnectivity, mean interconnectivity with correction factor (ratio between the average area and perimeter of mitochondria normalized to

their circularity) and mean elongation -, also designated as mitochondrial morphometrics, were quantified using Fiji software as previously reported [54].

3.8. Statistical Analysis

The results are expressed as a mean ± s.d. and analyzed using unpaired Student's two-tailed t-test (* $P < 0,05$; ** $P < 0,01$; *** $P < 0,001$).

4. Results

4.1. Role of LDL in the mitochondrial mass and network morphology of migrating TNBC cells

Previous studies have shown that LDL plays a key role in the aggressiveness of breast cancer, especially in the triple-negative subtype [19,27,29]. Preliminary results from our lab revealed that LDL exposure of TNBC cell is accompanied by an increase in the mitochondrial mass as determined by mtDNA content, MitoTracker staining and electron microscopy (Nóbrega-Pereira et *al*., unpublished). However, whether LDL-induced TNBC cells aggressiveness was due to mitochondrial metabolic adaptations as well as changes in mitochondrial dynamics, remained unclear and was addressed in this thesis.

In order to answer this question, we investigated a possible association between cell migration capacity and mitochondrial mass, and if this effect is exclusive for LDL or more broadly applies to other chemotaxis-inducing agents (capable of inducing cell migration). To do this, we exposed the human TNBC cell line MDA-MB-231 to LDL or lysophosphatidic acid (LPA), a potent chemoattractant reported as a cell migration inducer of several types of cancer cells, including TNBC [55] and performed a wound healing assay, followed by quantification of the mitochondrial mass by qPCR.

When MDA-MB-231 cells were exposed to LPA, there was a significant increase in the migratory capacity compared to control or LDL-exposed cells (**Figure 3a)** and **3b)**), however, this was not accompanied by a significant increase in the mitochondrial mass (**Figure 3c)**). These results suggest that the increase in mitochondrial mass of LDL-exposed MDA-MB-231 migrating cells may be specific to lipid-enriched signals and not to other chemotaxis-inducing agents. Indeed, preliminary data suggest that MDA-MB-231 cells exposed to palmitic acid (PA) have an increase in the migratory capacity, which is also accompanied by an increase in the mitochondrial mass (**Supplementary Figure 1**).

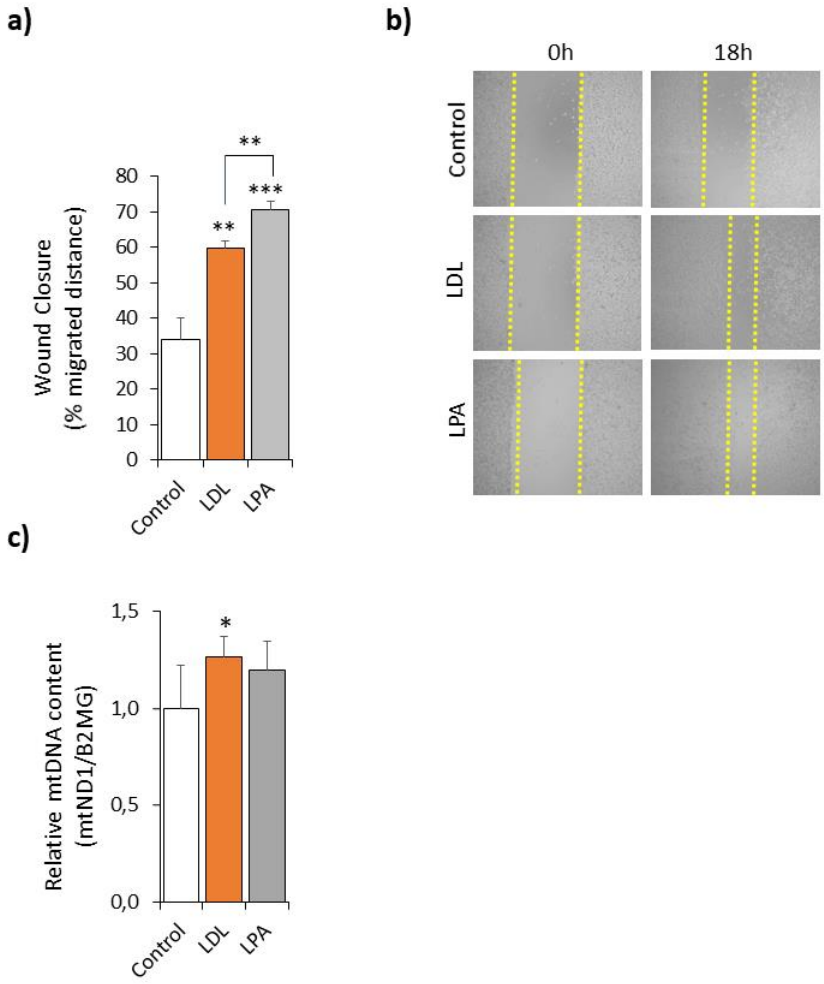


Figure 3. Effect of LDL and LPA in the migratory capacity and mitochondrial mass of MDA-MB-231 cells.

a) Migratory capacity represented as percentage of wound closure at 18h by wound healing assay (n=4/each from two independent experiments); **b)** Representative images of wound closure at 0h and 18h by optical microscopy (4x objective); **c)** Relative mtDNA content based on the mitochondrial *ND1* gene relative to the nuclear gene *B2MG* in LDL and LPA-exposed groups relative to the control group (n = 5/each from two independent experiments). Data are presented as mean ± s.d. Statistical significance was determined by two-tailed Student *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Taking into account these results and the knowledge that mitochondria are able to regulate their shape and structure in function of the microenvironment [30,33,34] we decided to analyze the mitochondrial network in migrating MDA-MB-231 under control or upon LDL-exposure by performing the wound-healing assay followed by immunofluorescence detection of the mitochondrial protein HSP60 using confocal microscopy.

We could observe two different phenotypes of mitochondrial network distribution: mitochondrial aggregates (clusters) around the nucleus, often located at only one side and designated *perinuclear mitochondria* (**Figure 4a**), **upper panel**) and long mitochondrial filaments, located unilaterally or bilaterally around the nucleus and distributed throughout the whole cell, designated as *filamentous mitochondrial* (**Figure 4a), lower panel**) [56]. Based on this observation, we decided to qualitatively analyze the type of mitochondrial network distribution between control and LDL-exposed MDA-MB-231 cells. We could observe that control MDA-MB-231 cells display only one phenotype, perinuclear mitochondria, while in the LDL-exposed cells the two phenotypes are observed, with approximately 40% filamentous and 60% perinuclear mitochondria cellular distribution (**Figure 4b)**).

Some studies relate perinuclear mitochondrial clustering to cell apoptosis [57,58], but unpublished data from Nóbrega-Pereira *et al*., revealed that there is no significant differences in apoptosis levels between control and LDL-exposed MDA-MB-231 cells, as determined by AnnexinV-7AAD staining. However, it is important to note that MDA-MB-231 cells under control conditions are cultured in lipid-depleted medium for 48h and this condition may affect mitochondrial network arrangement, since these organelles are very sensitive to changes in environment conditions, including changes in nutrients and oxygen availability[33]. As accessed for the mitochondrial mass with mtDNA content evaluation, it will be important to discern weather the effects of LDL in mitochondrial network distribution are unique or also present upon stimuli with other chemotaxis agents with lipidic (PA) or unrelated nature (such as LPA).

These results raised the possibility that LDL may regulate the shape and/or mitochondrial distribution in migrating cells which may have implications in the increased migratory capacity induced by LDL in TNBC cells.

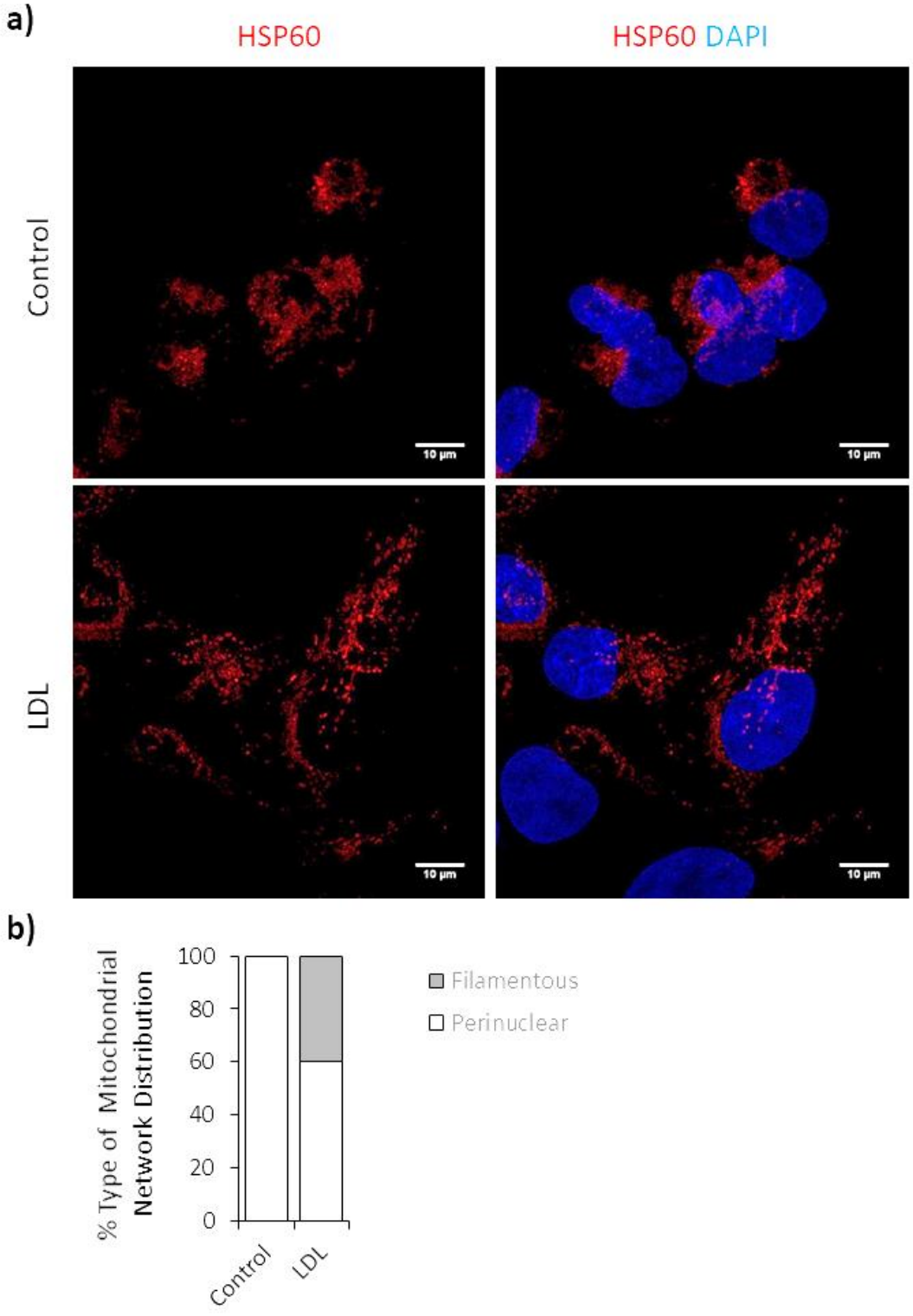


Figure 4. Effect of LDL in the mitochondrial network distribution of MDA-MB-231 migrating cells.

a) Representative confocal images of control (upper panel) and LDL-exposed (lower panel) MDA-MB-231 cells stained with HSP60 (mitochondria) and DAPI (nuclei). Scale bar equals 10 µm. **b)** Qualitative quantification of the % of mitochondria network distribution in migrating MDA-MB-231 cells (filamentous versus perinuclear; n=20/21 cells from control and LDL, respectively).

In order to obtain a more complete analysis of the mitochondrial network, including insights into mitochondrial dynamics, we perform a quantitative analysis of the parameters of mitochondrial morphology as previously reported by Wiemerslage and Lee [54]. The parameters of mitochondrial morphology, also known as mitochondrial morphometrics, were quantified using a macro developed by Ruben Dagda [59] for ImageJ software. This include the mean number and mean size of mitochondria, the interconnectivity (which describes the physical connections between mitochondria) and elongation, which describes the shape of mitochondria (a higher score for elongation means a higher level of fused mitochondria). On the interconnectivity parameter, we also introduced a correction factor to overcome the possibility of mitochondrial swelling, often observed in pathological conditions. Taken together, these parameters give an overview of the mitochondrial network distribution.

Thus, we quantified the mitochondrial morphometric parameters in MDA-MB-231 control or LDL-exposed after perform the wound-healing assay. In a study carried out by Zhao *et al*., the luminal-A breast cancer cell line MCF-7 presents larger mitochondria and increased mitochondrial length when compared to TNBC cell lines [31], therefore, we used the MCF-7 cell line as a control for the quantification method. Indeed, we were able to reproduce the published findings as we detected increase mitochondrial area in MCF-7 cells when compared to MDA-MB-231 cells (**Supplementary Figure 2**), as well as increased mitochondrial mass, which was also corroborated by us using MitoTracker staining (**Supplementary Figure 3**).

Concerning MDA-MB-231 cells, LDL-exposed cells have significantly more and larger mitochondria when compared to the control group (**Figure 5a)** and **5b)**). The interconnectivity parameter is also significantly altered between both conditions, with LDL-exposed MDA-MB-231 cells presenting approximately a 12-fold increase compared to control cells (**Figure 5c)**). As previously mentioned, interconnectivity describes the physical connections between mitochondria and is often associated with higher levels of fused mitochondria and, consequently less fragmented mitochondria. However, the elongation score obtained for LDL-exposed MDA-MB-231 cells does not support this statement.

According to Wiemerslage and Lee, if the elongation score is closer to 1, the mitochondria will be more circular and consequently the network will be more fragmented and, consequently, the less elongated; on the other hand values much higher than 1 describe mitochondria with a more abstract and elongated shape. [54] For MCF-7 and control and LDL-MDA-MB-231 cells, the elongation scores are all close to 1 and there are no significant differences among them (**Figure 5d)** and **Supplementary Figure 2**), which means that luminal A and TNBC cells exhibit fragmented mitochondria. This result is in line with previously reported data in

several cancer types, which often exhibit higher levels of mitochondria fragmentation due to increase expression of Drp1 and/or downregulation of Mfn2.

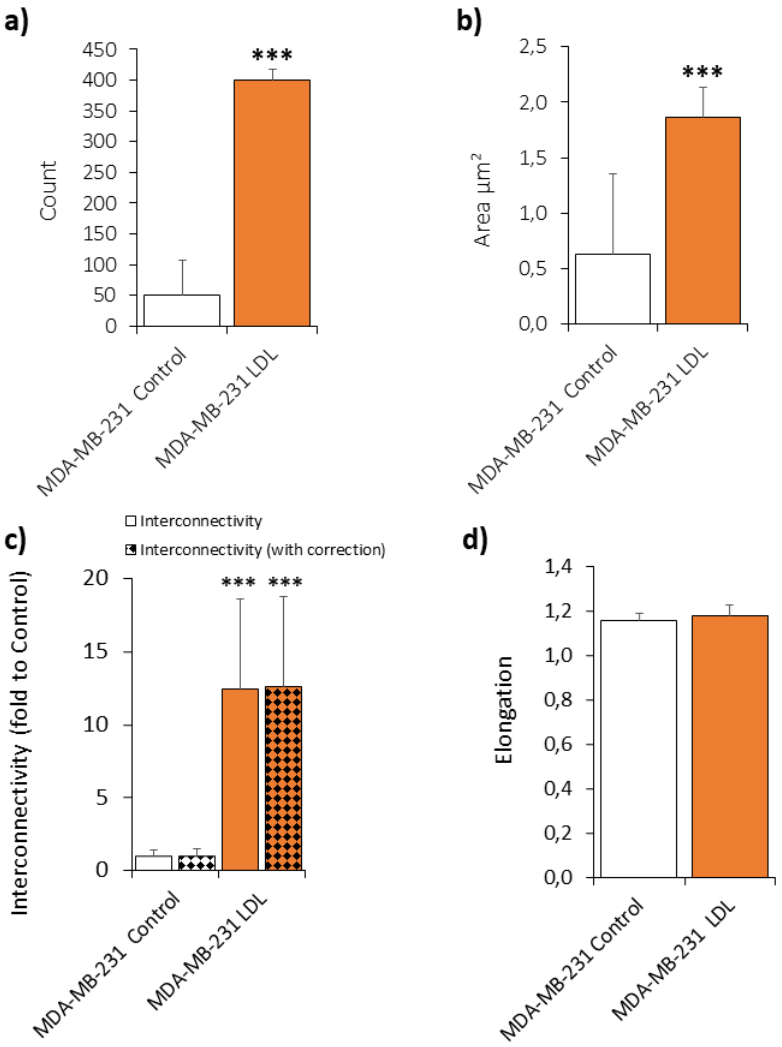


Figure 5. Effect of LDL in mitochondrial morphometrics in MDA-MB-231 migrating cells.

Quantification of mitochondrial morphometrics of Control and LDL-exposed MDA-MB-231 migrating cells (n ≥ 18 cells). **a)** Mean number of mitochondria; **b)** Mean size of mitochondria; **c)** Mean interconnectivity of mitochondria; **d)** Mean elongation of mitochondria. Data are presented as mean ± s.d. Statistical significance was determined by two-tailed Student *t*-test. **, $P < 0.01$; ***, $P < 0.001$

This quantitative analysis reveal similar results to other analytical techniques regarding mitochondria number (MitoTracker, mtDNA content), but inconsistent results in other parameters, namely the link between elevated mitochondrial interconnectivity and a higher fragmented network, which highlight the necessity of using several alternative quantitative

assays in order to have a more accurate determination of mitochondrial network dynamics and distribution across the cells.

In summary, this analysis revealed that LDL exposure influences the mitochondrial network morphology of MDA-MB-231 cells, namely increasing the number and size, as well as the physical connections established between mitochondria. On the other hand, LDL seams not to influence the elongation parameter which suggests that mitochondrial dynamics may not be altered.

4.2. Effect of LDL exposure in TNBC migrating cells under acute exposure to Taxol

Our results suggest that LDL-induced aggressiveness in TNBC cells, namely increased migratory capacity, is accompanied by alterations in the mitochondrial network (increased mitochondrial number, size, interconnectivity and filamentous distribution), therefore, we hypothesized that these alterations could impact the interaction between mitochondria and the cytoskeleton in migrating TNBC cells. In order to explore this possibility, we decided to disrupt the cytoskeleton dynamics using Taxol, a commonly used chemotherapeutic agent of the Taxane family. The mechanism of action of Taxol lies in the ability to stabilize microtubules and interfere in the dynamics of depolymerization and polymerization, consequently blocking mitosis and inducing apoptosis [40]. In addition, another effect reported by the action of Taxol is the formation of multinucleated cells, consisting of small nuclei resulting from mitotic chromosome segregation errors [41,60].

In order to evaluate the impact of LDL exposure in the effect of acute exposure to Taxol, we exposed MDA-MB-231 cells to Taxol alone (20 nM) or in combination with LDL and evaluated the number of binucleated and multinucleated cells. In Taxol-exposed MDA-MB-231 cells, the percentage of binucleated and multinucleated cells was about 20% and 40% (± s.d.), respectively (**Figure 6a) middle image** and **6b)**). On the other hand, adding LDL in combination with Taxol lead to a reduction (although non-significant) on the percentage of binucleated and multinucleated MDA-MB-231 cells to about 12% and 23%, respectively (± s.d.) (**Figure 6a) lower image** and **6b)**). Additionally, the regions of hyperpolymerized α-tubulin (regions with greater intensity of pixels) observed in Taxol exposed MDA-MB-231 cells (**Figure 6a) middle image**) where reduced when Taxol was combined to LDL. No significant alterations were observed in MDA-MB-231 control (**Figure 6a) upper)** or LDL-only exposed cells (data not shown).

For a more accurate analysis of the Taxol effect in MDA-MB-231 cells, we performed a cell death assay using AnnexinV-7AAD staining (**Figure 6c)**). When compared to the control condition, we could observe that LDL did not produced any effect, whereas Taxol significantly increased cell death. Moreover, when MDA-MB-231 were simultaneously exposed to Taxol and LDL, we could observe a rescue of the cell death, significantly lower regarding Taxol condition, but still significantly higher when compared to the control group.

Overall, we can conclude that the acute exposure to the cytoskeleton-destabilizing agent Taxol (at 20 nM) significantly increases cell death in Taxol-exposed MDA-MB-231 cells and compromises the cellular functional integrity, providing the desired effect for the subsequent experiments.

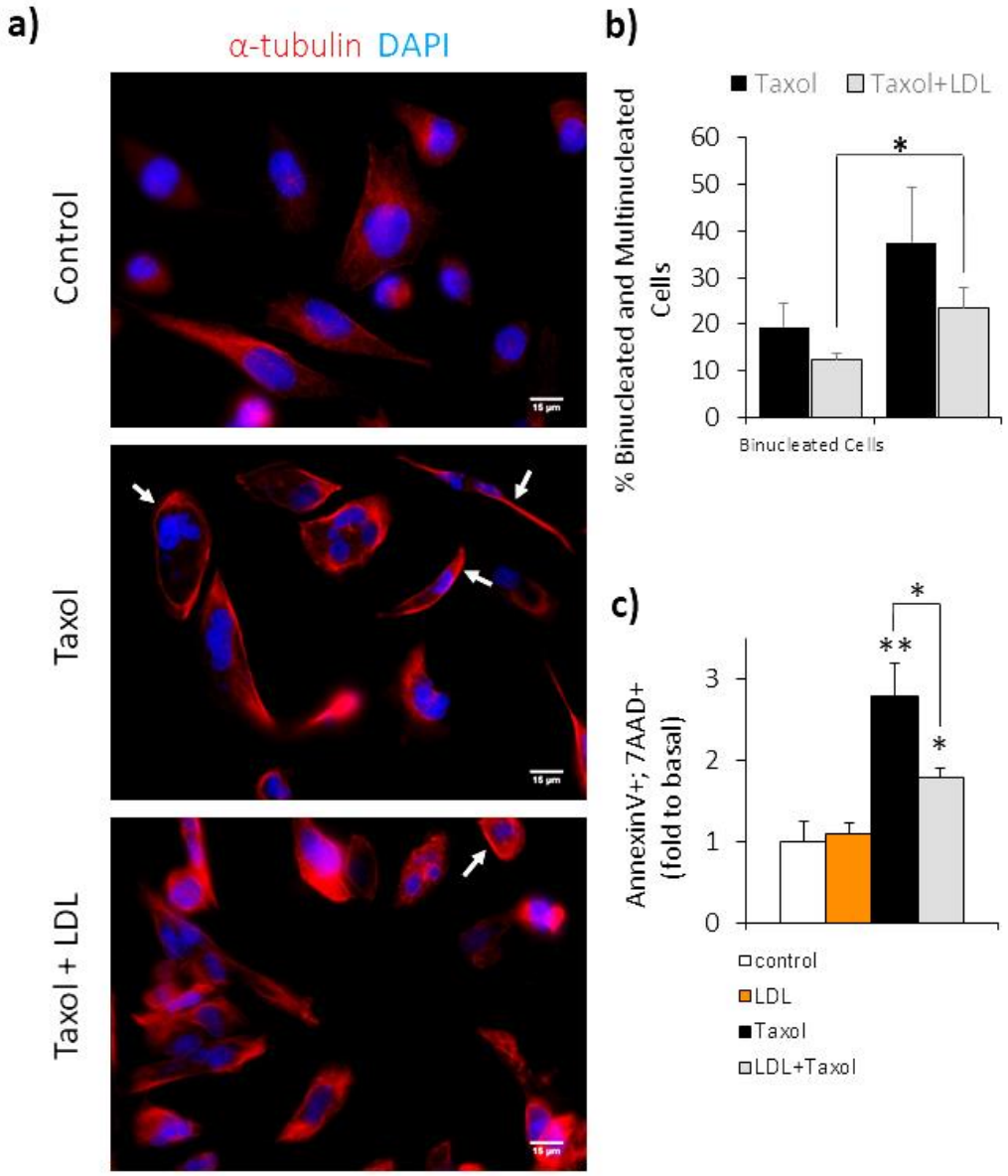


Figure 6. Effect of Taxol acute treatment alone and combined to LDL exposure in MDA-MB-231 cells.

a) Representative epifluorescence images of Control (upper image), Taxol 20 nM (middle image) and Taxol 20 nM + LDL exposed (lower image) of MDA-MB-231 cells stained with α-tubulin in red and DAPI (nuclei) in dark blue. White arrows indicate regions of higher pixel intensity that represent hyperpolymerized α-tubulin. Images were acquired with a 63x oil objective. Scale bar equals 10 µm. **b)** Percentage of binucleated and multinucleated MDA-MB-231 cells in the depicted conditions (n ≥ 100 cells each). Control and LDL-exposed MDA-MB-231 cells are not represented because they do not have bi and/or multinucleated cells. **c)** Cell death of MDA-MB-231 cells in the depicted conditions. Cell death assayed with AnnexinV-PE-7AAD staining by flow cytometry and analyzed using FlowJo software (n = 3 each). Data are presented as mean ± s.d. Statistical significance was determined by two-tailed Student *t*-test. *, $P < 0.05$; **, $P < 0.01$.

Next, we focused on our hypothesis, if the increased aggressiveness induced by LDL-exposure in MDA-MB-231 cells was mediated by specific interactions between the mitochondrial network and the cytoskeleton. To explore this question, we exposed MDA-MB-231 cells to LDL, Taxol (20 nM) or both and performed the wound-healing assay followed by quantification of the mitochondrial mass. We observed a significant decrease in the migratory capacity of MDA-MB-231 cells exposed to Taxol compared to the control group, whereas addition of LDL was able to revert the defect in the migratory capacity of Taxol-exposed MDA-MB-231 cells (**Figure 7a)** and **7b)**). The same trend is observed in the mitochondrial mass evaluated by qPCR of mtDNA content, where we could detect a significant decrease in the mitochondrial mass of Taxol-exposed MDA-MB-231 cells which was restored in the combined treatment of Taxol and LDL (**Figure 7c)**).

In order to more accurately determine the effect of Taxol treatment alone or in combination with LDL in the mitochondrial network distribution in MDA-MB-231 migrating cells, we performed the same quantification as before (**Figure 4b)**). For that, we exposed MDA-MB-231 cells to LDL, Taxol and both conditions and performed the wound-healing assay followed by immunofluorescence detection of HSP60.

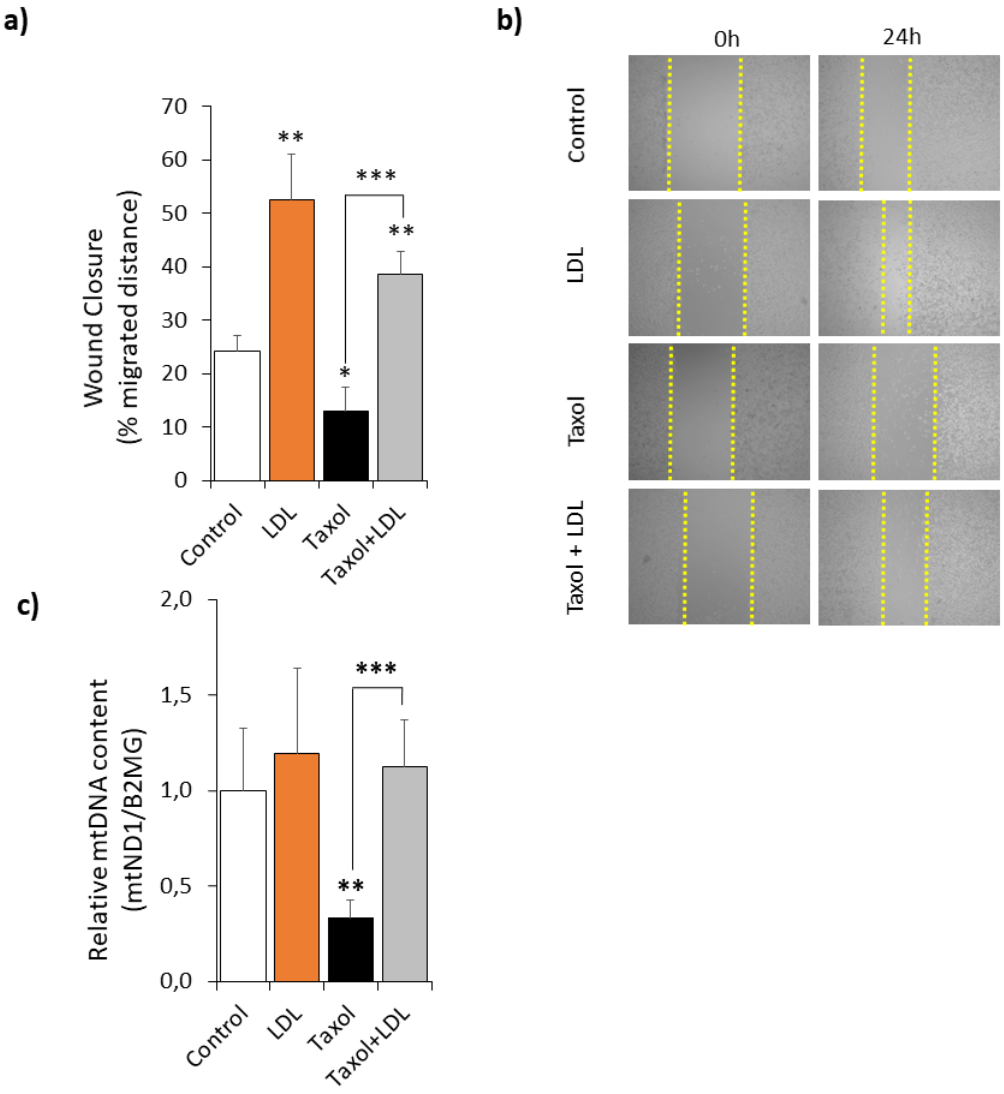
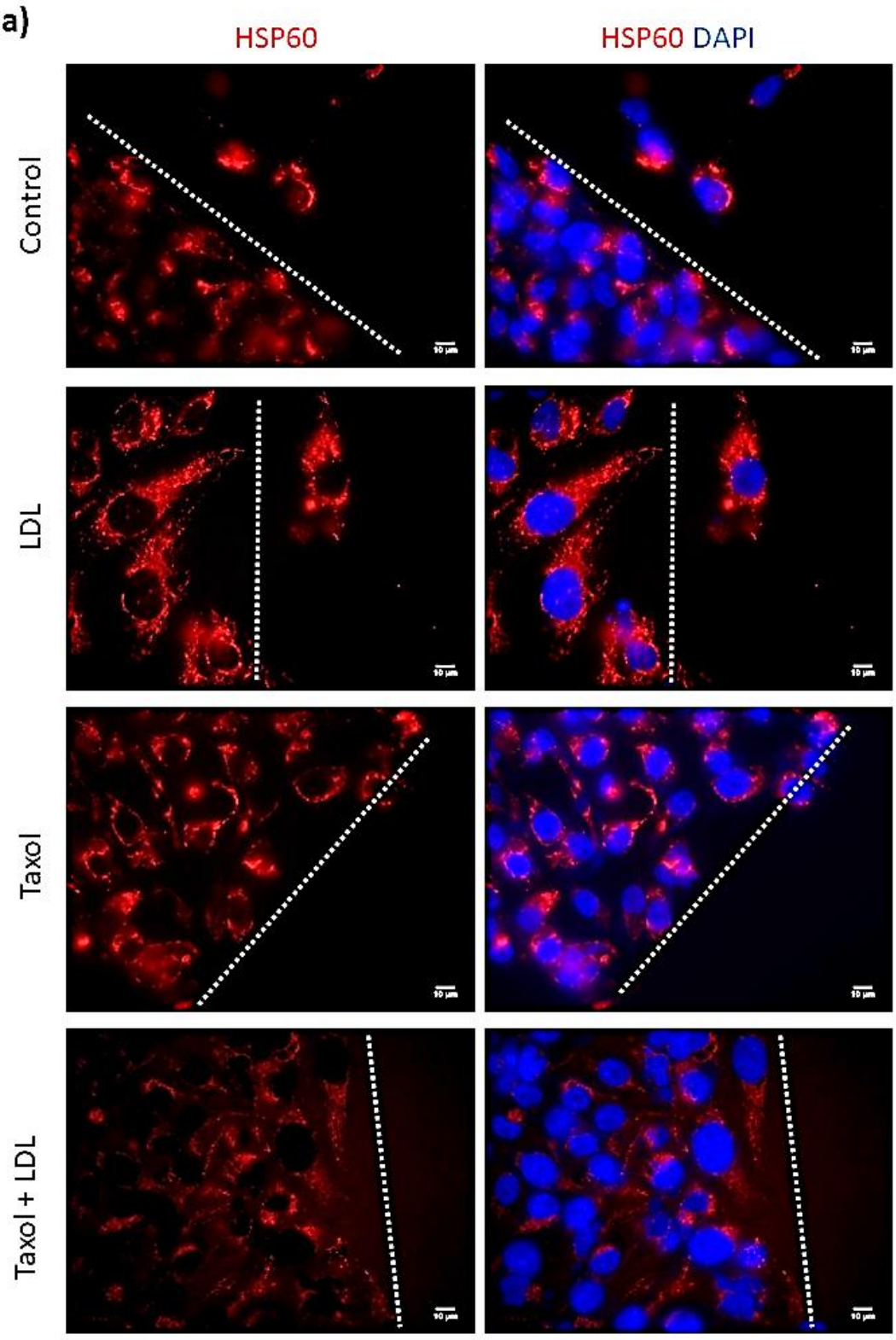


Figure 7. LDL reverts the migratory capacity and restores mitochondrial mass of MDA-MB-231 migrating cells upon acute exposure of Taxol.

a) Migratory capacity represented as percentage of wound closure at 24h by wound healing assay (n = 3/4 each) **b)** Representative images of wound closure at 0h and 24h by optical microscopy (4x objective) **c)** Relative mtDNA content based on the mitochondrial *ND1* gene relative to the nuclear gene *B2MG* (n = 5/6 from two independent experiments. Data are presented as mean ± s.d. Statistical significance was determined by two-tailed Student t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.


a)
HSP60
HSP60 DAPI
Control
LDL
Taxol
Taxol + LDL

b)

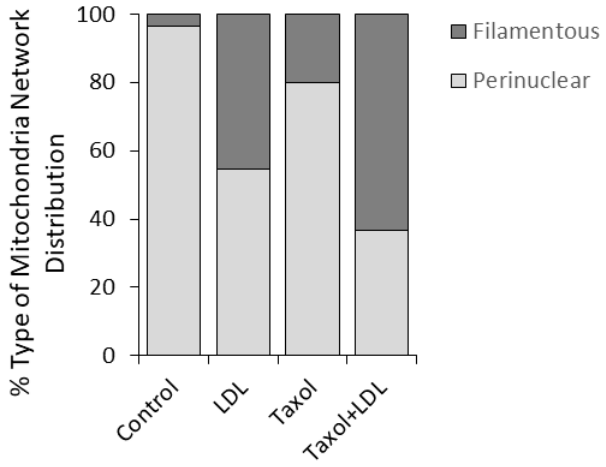


Figure 8. LDL reverts the phenotype induced by acute Taxol treatment in MDA-MB-231 migrating cells.

a) Representative epifluorescence images of Control (upper panel), LDL (second panel), Taxol 20 nM (third panel) and Taxol 20 nM+LDL exposed MDA-MB-231 cells (lower panel). Left column: HSP60 (mitochondria), right column: merge, HSP60 (mitochondria) and DAPI (nuclei). The dashed white line represents the wound front edge. Images were acquired with a 63x oil objective. Scale bar equals 10 µm. **b)** Qualitative quantification of mitochondria network distribution (filamentous *versus* perinuclear) (n=30/31 cells/each).

As previously shown (**Figure 4b)**), in MDA-MB-231 cells from the Control group, the perinuclear mitochondria phenotype is predominantly present (**Figure 8a) upper panel** and **8b)**) while in the MDA-MB-231 LDL-exposed cells, we observe both phenotypes with 55% perinuclear and 45% filamentous mitochondria distribution (**Figure 8a) second panel** and **8b)**). In regard to Taxol-exposed cells, it is mostly observed the perinuclear mitochondria network phenotype, just like in the control condition, although there is a slight decrease in the percentage of perinuclear mitochondria and a tenuous increase in the percentage of filamentous mitochondria. Moreover, when compared to LDL-exposed group, the Taxol-exposed cells exhibit a decrease in the percentage of filamentous mitochondria (around 20%) and an increase in perinuclear mitochondria (around 80%) (**Figure 8a**) **third panel** and **8b)**). Combined exposure of MDA-MB-231 cells to Taxol and LDL produced an increase in the percentage of filamentous mitochondria network distribution (approximately 65%) and a decrease in the percentage of perinuclear mitochondria to around 35% (**Figure 8a) lower panel** and **8b)**), when compared to the Taxol

condition. Thus, exposure of MDA-MB-231 cells to LDL was able to rescue the mitochondrial network distribution phenotype induced by Taxol, resembling the LDL-condition.

Overall, these results suggest, firstly, that cytoskeleton-destabilizing agent (in this case, Taxol) severely affect the migration of MDA-MB-231 that additionally display decreased mitochondrial mass (evaluated by mtDNA content) and disturbance of the mitochondrial network distribution; overall highlighting the importance of mitochondrial-cytoskeleton interactions in the migratory process of MDA-MB-231 cells. Moreover, we show that LDL is able to restore the defects in migratory capacity, mitochondrial mass (as evaluated by mtDNA content) and mitochondrial network distribution induced by the cytoskeleton-destabilizing agent Taxol, suggesting that LDL-induced migratory capabilities may indeed be strictly linked to mitochondrial-cytoskeleton interactions within MDA-MB-231 cells (**Supplementary Figure 4**).

4.3. Effect of LDL exposure in Taxol chronically-exposed TNBC

Abnormal lipid metabolism has been reported as a metabolic adapted feature of chemotherapy resistant breast cancer cells and several studies show that targeting metabolism of tumor cells could be a promising strategy to revert Taxol-resistance in several types of cancer, including TNBC.

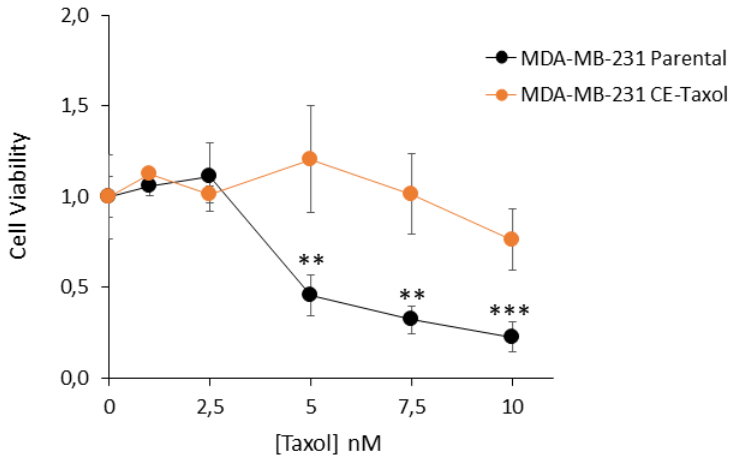
A major number of therapeutic guidelines recommend Taxol as first-line therapy for treatment of metastatic breast cancer [39,42,61,62], due to its ability to suppress cancer cells migration and proliferation [63]. In the clinic, patients with advanced breast cancer and bone metastasis present higher response rates after treatment with Taxol [64], evidencing the role of Taxol in impairing and compromising cancer cells migration.

We wanted to address the impact of chronic exposure to the chemotherapeutic and cytoskeleton-destabilizing agent Taxol in the migratory capability and mitochondrial mass of MDA-MB-231 cells and the impact of higher LDL environments on these parameters.

We started by exposing two TNBC cell lines, MDA-MB-231 and MDA-MB-468, chronically to Taxol with the aim of establishing Taxol-resistant cell lines. There is a variety of protocols with different approaches to establish Taxol-resistant BC cell lines [52,65,66]. The values of IC_{50} reported for different cancer cell lines are drug and time dependent and for this reason, there are some discrepancies between the values of different studies. We decided to expose TNBC cell lines to a chronic concentration of Taxol within the range of IC_{50} values reported for the MDA-MB-231 and MDA-MB-468 cell lines. The reported IC_{50} values varies between 1 - 9 nM for MDA-MB-231 cells and between 1,8 nM - 4 nM for MDA-MB-468 [42,67–69].

Thus, in order to establish Taxol-resistant TNBC cell lines, we exposed MDA-MB-231 and MDA-MB-468 chronically to 2,5 nM Taxol during 12 weeks. After this period, we evaluated the Taxol-sensitivity by performing a viability assay under increasing Taxol doses (**Figure 9**).

a)



b)

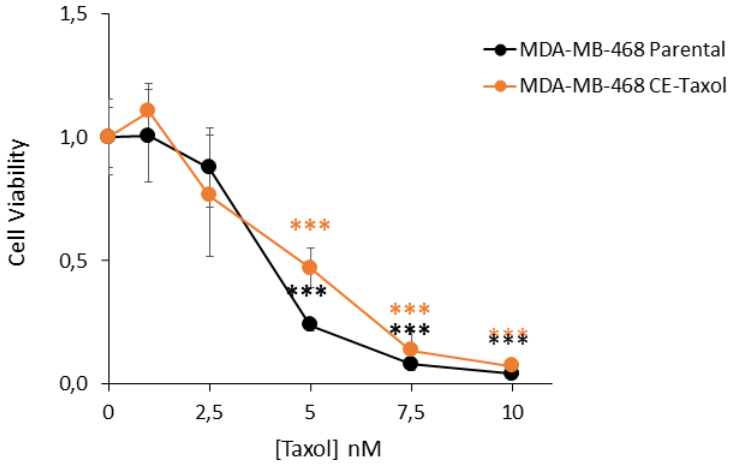


Figure 9. Cell viability of human TNBC (MDA-MB-231 and MDA-MB-468) parental and Chronic Exposed to Taxol (CE-Taxol) cell lines.

To generate Taxol-resistant TNBC cell lines, MDA-MB-231 and MDA-MB-468 were exposed to a chronic concentration of Taxol 2,5 nM during 12 weeks. To evaluate the sensitivity to Taxol, we perform a viability assay with MDA-MB-231 Parental and CE-Taxol (**a)** and MDA-MB-468 Parental and CE-Taxol (**b)** under different Taxol concentrations (0 nM, 1 nM, 2,5 nM , 5 nM, 7,5 nM and 10 nM) during 5 days. Cell viability was measured by counting the number of viable cells in the fifth day using Trypan Blue exclusion test by hemocytometer counts (4 squares per well, n=4 each from one representative experiment). Data are presented as mean ± s.d. Statistical significance was determined by two-tailed Student t-test. **, $P < 0.01$; ***, $P < 0.001$.

Regarding MDA-MB-231 CE-Taxol cells, it should be noted that for concentrations greater than 5 nM, the parental MDA-MB-231 cell line exhibits significantly higher sensitivity and lower cell viability compared to concentrations of 1 and 2,5 nM (**Figure 9a)**). We could say that at the end of 12 weeks of chronic exposure, cells have acquired resistance to Taxol for concentrations that range between 5 nM and 10 nM, evidenced by their lower sensitivity and higher cell viability when compared to the parental cell line. Moreover, we do not observe differences in cell viability between untreated and Taxol treated (for any dose) MDA-MB-231 CE-Taxol cells. On the other hand, after 12 weeks of chronic exposure, there is no difference in the Taxol-sensitivity of MDA-MB-468 CE-Taxol when compared to the parental cell line, since both lines present significantly increased sensitivity and lower cell viability for concentrations higher than 5 nM (**Figure 9b)**), which probably means that the time of Taxol-exposure (12 weeks) was not enough to induce resistance or to decrease sensitivity in this cell line.

Overall, we can conclude that after 12 weeks exposure, we were able to establish a MDA-MB-231 cell line with acquired resistance to Taxol, as determined by its lower sensitivity to the drug for concentrations between 5 and 10 nM, but still displaying sensitivity at 10 nM, therefore we decided to designate as Taxol chronic-exposed (CE) MDA-MB-231 rather than Taxol-resistant cells.

Next, we analyzed the impact of Taxol-chronic exposure in the parameters of TNBC cell lines aggressiveness (migration and proliferation) and mitochondrial mass and whether LDL-enriched environments could modulate these parameters. For that, we exposed CE-Taxol MDA-MB-231 cells to LDL, Taxol (2,5 nM; the dose used for the chronic exposure) and both in combination and performed the wound-healing assay, followed by quantification of mitochondrial mass and additionally performed a proliferation assay under the same conditions (**Figure 10**). Additionally, in order to address if the effect produced by LDL exposure was similar between parental and chronically-exposed cell lines, we also performed a wound-healing assay, followed by quantification of mitochondrial mass, as well as a proliferation assay upon control and LDL conditions (**Figure 11**).

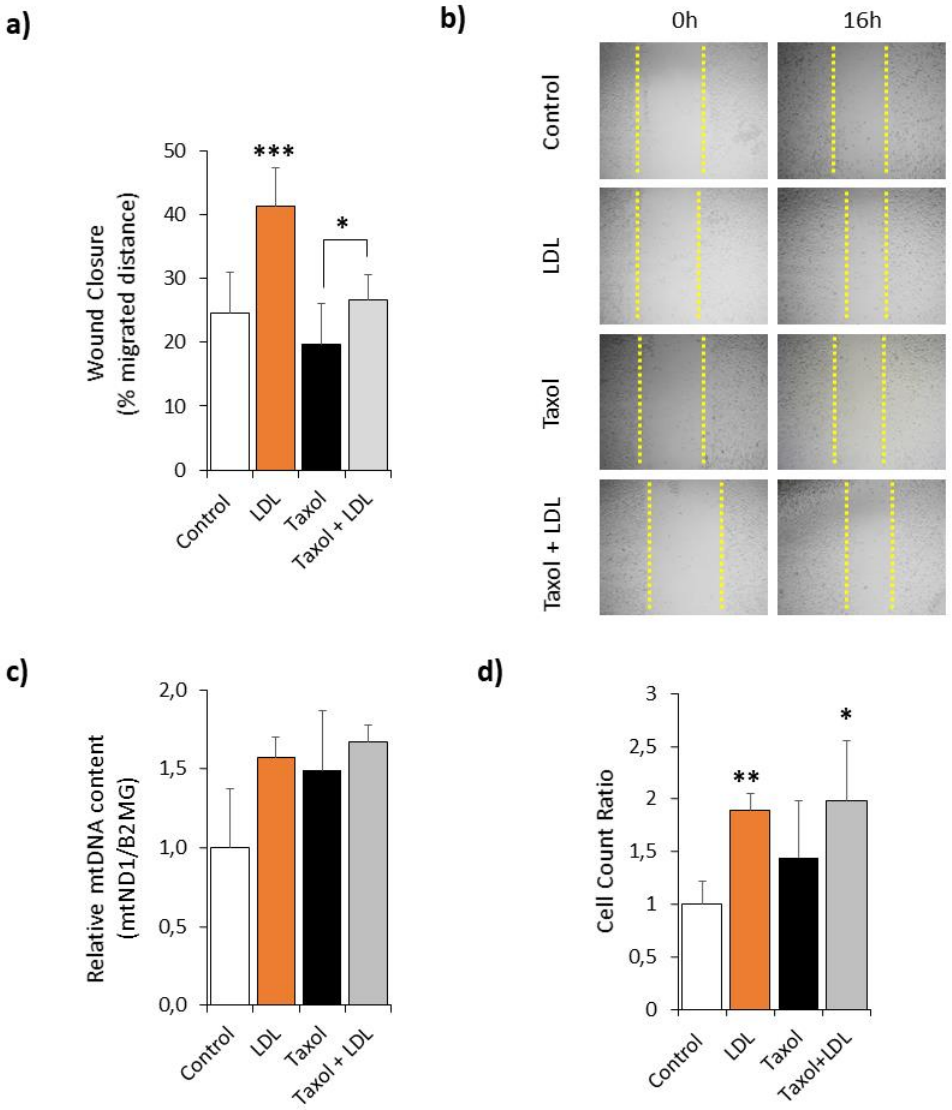


Figure 10. Effect of LDL in the migratory, proliferation and mitochondrial mass of MDA-MB-231 CE-Taxol cells.

a) Migratory capacity represented as percentage of wound closure at 16h by wound healing assay (n = 4 each from one representative experiment); **b)** Representative images of wound closure at 0h and 16h by optical microscopy (4x objective); **c)** Relative mtDNA content based on the mitochondrial *ND1* gene relative to the nuclear *B2MG* gene (n = 3 each) **d)** Proliferation assay by cell count ratio. The number of viable cells was counted using Trypan Blue exclusion test by hemocytometer counts (4 squares per well, n=4 each). Data are presented as mean ± s.d. Statistical significance was determined by two-tailed Student t-test. **, $P < 0.01$; ***, $P < 0.001$.

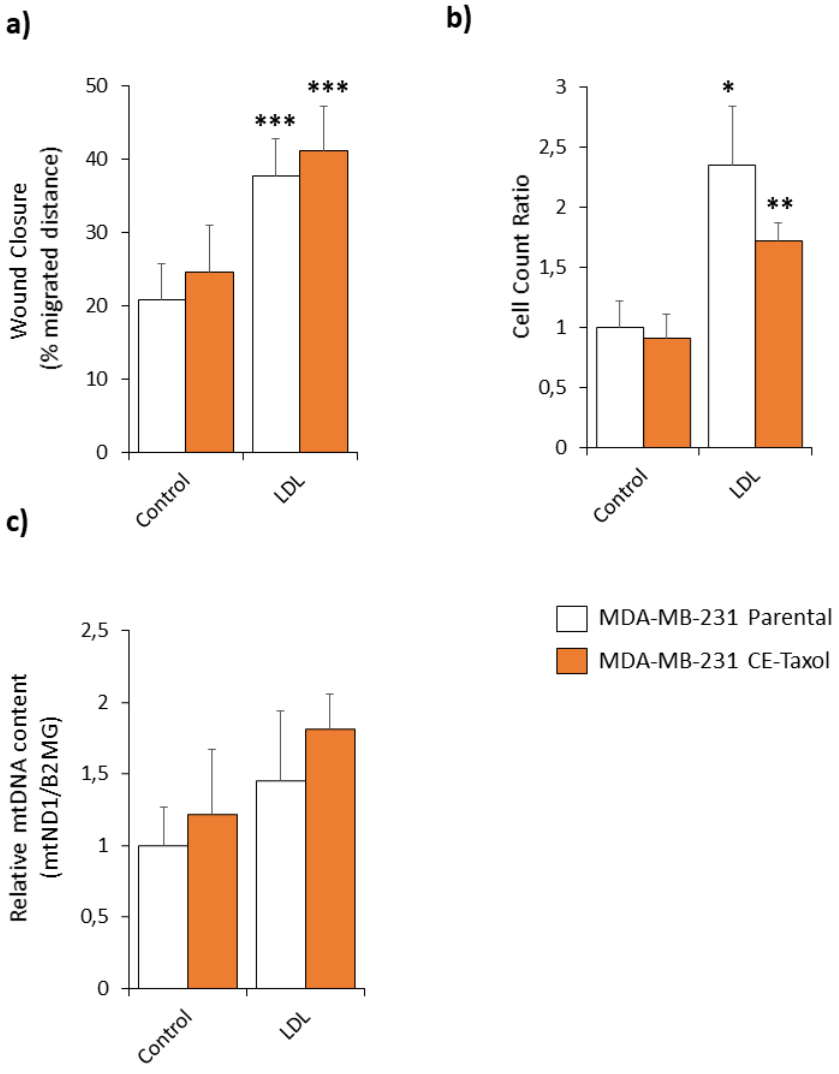


Figure 11. Analysis of the effect of LDL between MDA-MB-231 Parental and MDA-MB-231 chronic exposed to Taxol 2,5 nM (MDA-MB-231 CE-Taxol).

a) Migratory capacity represented as percentage of wound closure at 16h by wound healing assay (Control, LDL, n = 4, each); **b)** Proliferation assay by cell count ratio where the number of viable cells was counted in the second day using Trypan Blue exclusion test by hemocytometer counts (Control, LDL, 4 squares per well, n=4 each); **c)** Relative mtDNA content based on the mitochondrial *ND1* gene relative to the nuclear *B2MG* gene in LDL-exposed group relative to the control group (n=3/4). Data are presented as mean ± s.d. Statistical significance was determined by two-tailed Student *t*-test. *, $P <$ 0,05 **, $P < 0.01$; ***, $P < 0.001$.

Similarly, to what occurs with the MDA-MB-231 parental line (**Figure 7a)** and **b)**), LDL significantly increases the migratory (**Figure 10a)** and **10b)**) of MDA-MB-231 CE-Taxol cells and the same trend is observed for the mitochondrial mass (**Figure 10c)**). Additionally, LDL exposure also induced a significant increase in proliferation capacity in MDA-MB-231 CE-Taxol cells (**Figure 10d)**). Therefore, we comparing both parental and Taxol chronic-exposed MDA-MB-231 cells, we detected no significant differences in the LDL induced phenotype (**Figure 11**). In other words, the values for increased cell migration and proliferation (**Figure 11a)** and **c)**) and increased mitochondrial mass (**Figure 11b)**) are similar for both control and LDL conditions in MDA-MB-231 parental and MDA-MB-231 CE-Taxol.

We also could observe that, contrary to what was observed upon acute exposure of Taxol 20 nM (**Figure 7a)** and **7b)**) in parental MDA-MB-231 cells, treatment with a low dose of Taxol does not affect the migratory capacity or mitochondrial mass of MDA-MB-231 CE-Taxol (**Figure 10a)** and **10b)**), resembling the control levels. Also, low Taxol doses do not interfere with the proliferation capacity of MDA-MB-231 CE-Taxol (**Figure 10d)**). Thus, even in the presence of a low dose of cytoskeleton-destabilizing agent, Taxol, MDA-MB-231 CE-Taxol cells do not exhibit defects in the migratory and proliferation capacity.

Regarding MDA-MB-231 CE-Taxol cells, we observe that the simultaneous addition of Taxol and LDL does not cause a very marked increase over the Taxol condition, except for the migratory capacity in which a slight but significant increase is observed upon combined treatment, although it did not reach the increase percentage displayed by LDL-treated MDA-MB-231 CE-Taxol cells.

Therefore, preliminary results suggest that MDA-MB-231 CE-Taxol has no defects on the migratory and proliferative capacity nor in mitochondrial mass and the parameters induced by LDL exposure in MDA-MB-231 cells are largely retained in MDA-MB-231 with acquired resistance to Taxol except for the migratory capability. However, in MDA-MB-231 CE-Taxol, LDL was not able to fully recover the defect produced by a low dose of Taxol in the migratory capacity, contrary to what had been observed with the addition of an acute dose of Taxol combined with LDL in untreated MDA-MB-231 cells (**Figure 7a)** and **b)**). Moreover, we can preliminarily conclude that chronic exposure to Taxol seems to produce a permanent defect in cell motility that cannot be rescued by LDL exposure.

5. Discussion

As observed in other types of cancer, TNBC cells are able to reprogram their cellular metabolism (including lipid metabolism) in communication with the surrounding microenvironment, in order to successfully met the requirements that sustain cell growth, proliferation and invasion capabilities [16,19–21].

A major focus of our laboratory is to study the impact of the tumor micro- and macro-environment, especially the role of lipids availability, in the acquisition of aggressive tumors phenotypes in breast cancer. In particular, it was recently shown that LDL plays a key role in TNBC growth and proliferation [27,29]. More recently, it was observed that LDL-exposed TNBC cells present increased mitochondrial mass as determined by MitoTracker staining, quantification of mtDNA content and electron microscopy (Nóbrega-Pereira *et al*., unpublished). Despite this information, the exact nature of mitochondrial network adaptations induced by LDL-exposure and how it affects the acquisition of an aggressiveness migratory phenotype remains unclear.

In this context, one of the aims of this project was to understand how LDL-exposure impacts the mitochondrial phenotype in TNBC migrating cells, including mitochondrial mass and mitochondrial network distribution and morphology.

LDL-exposure increases cell migration and proliferation in TNBC cells [19], which are accompanied by changes in mitochondrial mass and arrangements in mitochondrial network, as suggested by our results. Moreover, our data suggest that changes in mitochondrial mass observed during cell migration may be specific from lipid-enriched environments, since LPA-induced migration did not affected mitochondrial mass contrary to what was observed in LDL and PA-exposed migrating TNBC cells. This observation is not surprising since one study reported an association between cancer aggressiveness (such as increased cell proliferation, migration and metastasis) and expression of a scavenger receptor, CD36, which may uptake LDL and different types of fatty acids.[70] Nevertheless, the exact molecular nature of the mechanisms present in both LDL and PA that promotes the effects in MDA-MB-231 cells was not assessed in this study.

Mitochondria are versatile organelles capable of modulate their shape and size depending on the cell requirements and in interaction with the micro- and macro- environment [33,34].

Depending on the cell type and the exact mitochondria program most active in a certain moment, mitochondria can exhibit different network arrangements [56]. In this study, we observed two different arrangements of mitochondrial network: mitochondrial aggregates (clusters) located preferentially at only one side of the nucleus – *perinuclear mitochondria* - and

long filaments, located randomly unilaterally or bilaterally around the nucleus and distributed throughout the whole cell – *filamentous mitochondria.*

In LDL-exposed cells, MDA-MB-231 cells exhibit both phenotypes. The presence of MDA-MB-231 cells at the front of the wound displaying a filamentous arrangement could be associated with the increased migratory capacity observed in this condition. In line with these observations, it was shown by others that in migrating TNBC and PC3 (human prostate cancer) cells, the presence of mitochondria between the nucleus and the leading edge of the cell is associated with higher velocity of migration and increased directional persistence. This study raises the possibility that a great mitochondrial density at the leading edge of the cell is essential in order to sustain the occurrence of high energetic processes, including actin polymerization and the formation of new focal adhesions, which are essential for cell motility[36]. Another recent study revealed that increased mitochondria fission is required for the accumulation of mitochondria in the lamellipodia region of migrating TNBC cells, a key step for cell motility and BC cells migration and invasion [31]. This study evidences the importance of mitochondrial network arrangement throughout the whole cell, especially at the leading edge, to enhance metastatic potential in tumor cells. Additionally, it was reported that PI3K-inhibitors induce signaling reprogramming in tumor cells and as a consequence, mitochondria are moved to the leading edge of the cell where important processes for cancer progression are occurring, such as lamellipodia and focal adhesion formation and random cell motility [71].

We went further to study mitochondrial network morphology using ImageJ software and our quantitative analysis showed that LDL exposure in MDA-MB-231 cells increases mitochondria number, size and interconnectivity, but not elongation.

Overall, our results suggest that LDL could regulate the shape and the mitochondrial network distribution, inducing a more filamentous arrangement, which may be implicated in the increased migratory capacity induced by LDL-exposure in TNBC cells. Moreover, this aggressiveness induced by LDL in MDA-MB-231 cells is accompanied by changes in mitochondrial morphology, namely increased mitochondria number, size and interconnectivity.

We hypothesized if the alterations mentioned above could be due to a differential interaction between mitochondria and the cytoskeleton in TNBC migrating cells upon LDL-exposure. In order to address this question, we decided to use Taxol, a widely used chemotherapeutic and cytoskeleton-destabilizing drug, to interfere and disrupt cytoskeleton dynamics.

Our data show that exposure to an acute dose of Taxol adversely impacts the migration of MDA-MB-231 cells, where we could observe a decrease in mitochondrial mass (as determined by evaluation of mtDNA content) and a disruption in the mitochondrial network, evidencing the

key role of mitochondrial-cytoskeleton interaction during MDA-MB-231 migration. Interestingly, LDL seems to be capable to reverse the defects caused by Taxol-exposure, namely migratory capacity, mitochondrial mass and mitochondrial network distribution.

Mitochondria are able to readapt their dynamics and distribution in a way that favors cell the most, both under physiological and pathological conditions including cancer. Indeed, in TNBC cells, mitochondria change from a perinuclear distribution to a filamentous arrangement distributed all over the leading edge of the cells during the process of lamellipodia formation and in close contact to F-actin filaments. In agreement, we also observe increase lamellipodia formation in LDL-exposed TNBC migrating cells (**Supplementary Figure 5**). This process is essential for cancer invasion and progression and requires a great number of fragmented mitochondria [31].

Anesti and Scorrano reviewed the importance of cytoskeletal proteins in modulating the shape and distribution of the mitochondrial network across the cell [34], for instance ablation of certain cytoskeleton proteins could be accompanied by a specific rearrangement of the mitochondrial network that leads to cell apoptosis [57]. This data enhanced the essential role of the interaction between mitochondria and cytoskeleton to the cell. Since tumor cells are permanently exposed to high levels of cellular stress, the cytoskeleton-mitochondria interaction will be crucial for malignant cells to be able to adapt to different environments that tumor cells encounter, and rearrange the mitochondrial network in a way that favors tumor progression. A very recent study in HeLa cells, revealed that Miro1/2, both outer mitochondrial membrane proteins, directly bind to myosin XIX (Myo19) and downregulation of Myo19, leads to perinuclear clustering of mitochondria [58] .

During cancer cells migration and invasion, fragmented mitochondria appear to be a requirement for efficient mitochondria relocation to specific cell sites. Organelles transport within the cells is a process majority mediated by cytoskeleton motor proteins; therefore, mitochondria fragmentation could be a prerequisite for better interaction with cytoskeletal proteins, which in turn mobilize mitochondria to cell sites with high-energy demand, including lamellipodia formation areas.

Our mitochondrial morphometrics analysis revealed that MDA-MB-231 exhibit highly fragmented mitochondria network (more fission). Additionally, in migrating TNBC cells, LDL exposure restored the migratory capacity, as well as the mitochondrial mass and network distribution disturbance induced by the cytoskeleton-destabilizing drug Taxol. Therefore, LDL-induced aggressiveness phenotype, namely increased migratory capacity, may rely in specific mitochondrial-cytoskeleton interactions.

Since reprogramming cell metabolism is one of the most common adaptations displayed by chemotherapy resistant cancer cells, in the last aim of this project we decided to explore the impact of chronic-exposure to the chemotherapeutic agent Taxol in the migratory capacity and mitochondrial mass of TNBC cells and how exposure to LDL environments could affect these parameters.

In hepatocellular carcinoma (HCC), upregulation of STAR, an essential protein that regulates cholesterol import to mitochondria, contributes to higher levels of mitochondrial cholesterol content and decreased sensitivity to chemotherapeutic agents. Additionally, the same study demonstrated that knockdown of STAR resensitized HCC cells to chemotherapy[72]. Some studies report the use of statins as anti-cancer drugs and their combined use with chemotherapeutic agents exhibit synergistic effects, reducing tumor growth and proliferation[73], as well as reduce the development of multidrug resistance[74]. In breast cancer, the use of statins is associated with lower incidence of relapses in patients diagnosed at early stage[75] and with lower acquired resistance to endocrine therapy in patients with ER+ breast cancer[76].

Although clinical studies show a therapeutic benefit in the combination of statins and chemotherapeutic drugs, there is no clear evidence about the molecular mechanisms underlying the possible link between hypercholesterolemia and resistance to chemotherapy. In this part of the study, we wanted to address how chronic-exposed TNBC cells react to high cholesterol environments, namely in terms of migration and proliferation capacity and mitochondrial mass.

Our results show that MDA-MB-231 CE-Taxol cells present no defects in migration and proliferation capacity, or in the mitochondrial mass, even in the presence of a low dose of Taxol, when compared to untreated (control) MDA-MB-231 CE-Taxol cells. Moreover, we could observe that parental MDA-MB-231 and MDA-MB-231 CE-Taxol cells, both exhibit similar responses to LDL-exposure in terms of proliferation and migration capacity and mitochondrial mass.

Since Taxol chronic-exposed MDA-MB-231 cells present unaltered proliferation and migration capability and unchanged mitochondrial mass, we can preliminary conclude that acquired resistance to Taxol could be (somehow) dependent on mitochondrial activity.

It was reported in lung adenocarcinoma that cells resistant to cisplatin, a chemotherapeutic agent, exhibit increased mitochondrial membrane potential (MMPs), which is accompanied with increased migratory and invasive capacity [77]. In line with our observations, this study suggests that adaptations in mitochondria could be a determinant step to tumor cells acquire drug-resistance.

In another study, evaluation of mitochondrial mass was used as a biomarker of chemotherapy sensitivity in HeLa cells. Levels of apoptotic proteins are modulated by the

number of mitochondria and increased mitochondrial mass is associated with more susceptibility to the apoptotic machinery, which in turn induces greater sensitivity to chemotherapeutic agents in these cells [78]. Interestingly (and in line with our observations), in MDA-MB-231 and MCF-7 cells, a TNBC and a Luminal A breast cancer cell lines, respectively, it was shown that increased mitochondrial mass is associated with a stem-like phenotype, which in turn induces less sensitivity to Taxol[79]. In general, both studies have demonstrated that depending on the type of cancer and the stage of the disease, chemotherapy resistance could be accompanied by adaptations in mitochondria, which in turn may be determinant for the drug-resistant phenotype.

Exposure of MDA-MB-231 CE-Taxol to low doses of Taxol, did not produced major changes in proliferation, migration and mitochondrial mass compared to MDA-MB-231 CE-Taxol untreated. However, when combined to a low dose of Taxol, LDL interestingly did not significantly alter this fact (except for the migratory capacity, where we could observe a slight but significant increase when compared to MDA-MB-231 CE-Taxol untreated and treated with Taxol 2,5 nM).

Therefore, these preliminary data suggest that, in spite of mtDNA levels between MDA-MB-231 CE-Taxol exposed to LDL or MDA-MB-231 CE-Taxol exposed to LDL and Taxol remain similar, low doses of Taxol treatment in MDA-MB-231 CE-Taxol cells can compromise the cytoskeleton dynamics that, in turn, do not allow LDL treatment to rescue the migratory capacity.

Taken together, this study provides new highlights about the role of the aggressiveness phenotype induced by LDL exposure.

Firstly, LDL-induced increased migration capability is accompanied by changes in several mitochondrial morphology parameters, as well as network arrangement, which in turn seems to be dependent on specific mitochondria-cytoskeleton interactions. Thus, targeting mitochondria-cytoskeleton interaction could be a promising strategy to disrupt tumor cells invasion and metastasis. However, further studies are necessary to determine the exact molecular mechanisms underlying this interaction.

Regarding Taxol-resistance, our preliminary data suggest that acquired resistance to Taxol (or less sensitivity) could be somehow dependent on the mitochondrial activity. Moreover, our preliminary results may unravel the role of LDL in the context of chemotherapy resistance, which it is not a much elucidated research field. After Taxol-treatment, LDL is able to severely increase cell proliferation and migration, enhancing the metastatic and invasive potential of these cells, whereas during Taxol treatment this effect is not so pronounced. In other words, during Taxol treatment the aggressiveness features induced by LDL exposure are attenuated in some manner.

However, after treatment with Taxol, tumor cells in the presence of a LDL-enriched environment are able to recuperate their metastatic and proliferative potential.

Overall, these findings provide novel ideas about the role of LDL in cancer aggressiveness that could be extremely important in the clinic, more precisely in terms of acquired resistant to chemotherapy. Moreover, LDL appears once again as an interesting candidate to target and, consequently, to disrupt cancer progression.

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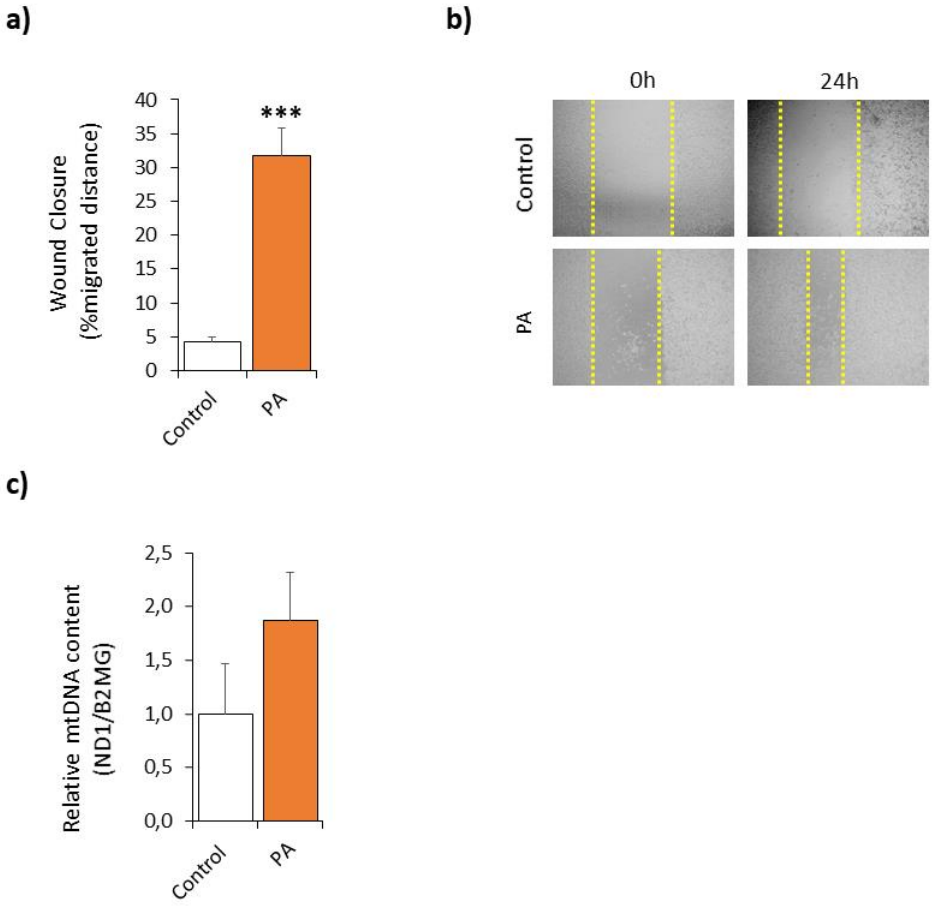
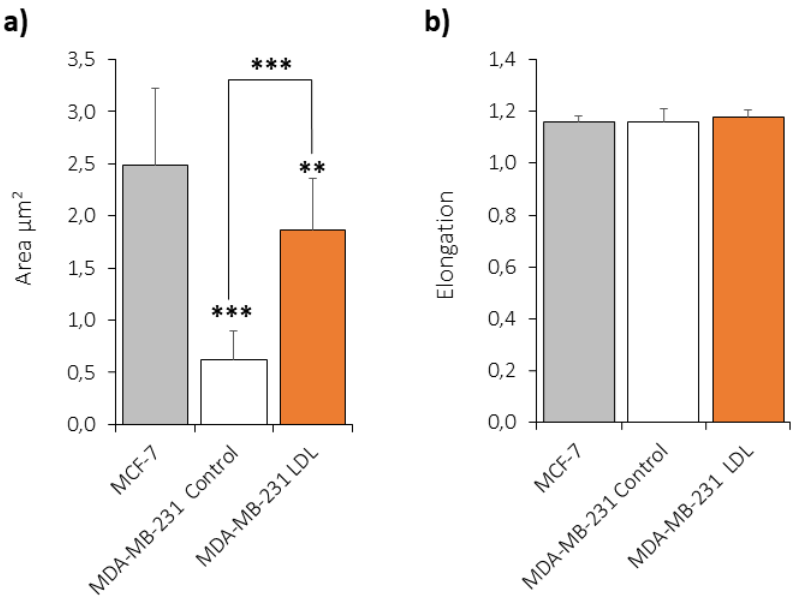
7. Supplementary Data



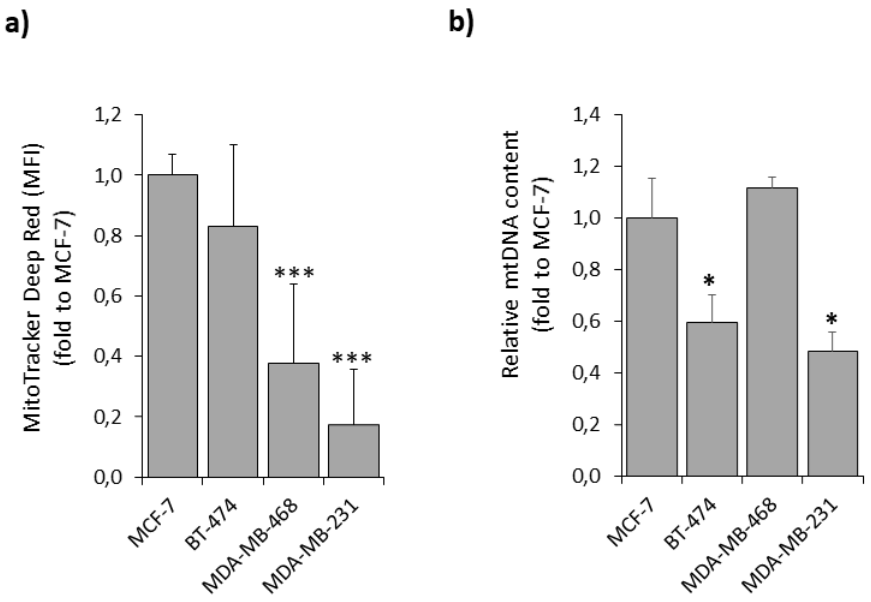
Supplementary Figure 1. Effect of PA in the migratory capacity and mitochondrial mass of MDA-MB-231 cells.

a) Migratory capacity represented as percentage of wound closure at 24h by wound healing assay (n=3); **b)** Representative images of wound closure at 0h and 24h by optical microscopy (4x objective); **c)** Relative mtDNA content based on the mitochondrial *ND1* gene relative to the nuclear gene *B2MG* in PA-exposed group relative to the control group (n =4). Data are presented as mean ± s.d. Statistical significance was determined by two-tailed Student *t*-test. ***, $P < 0.001$.



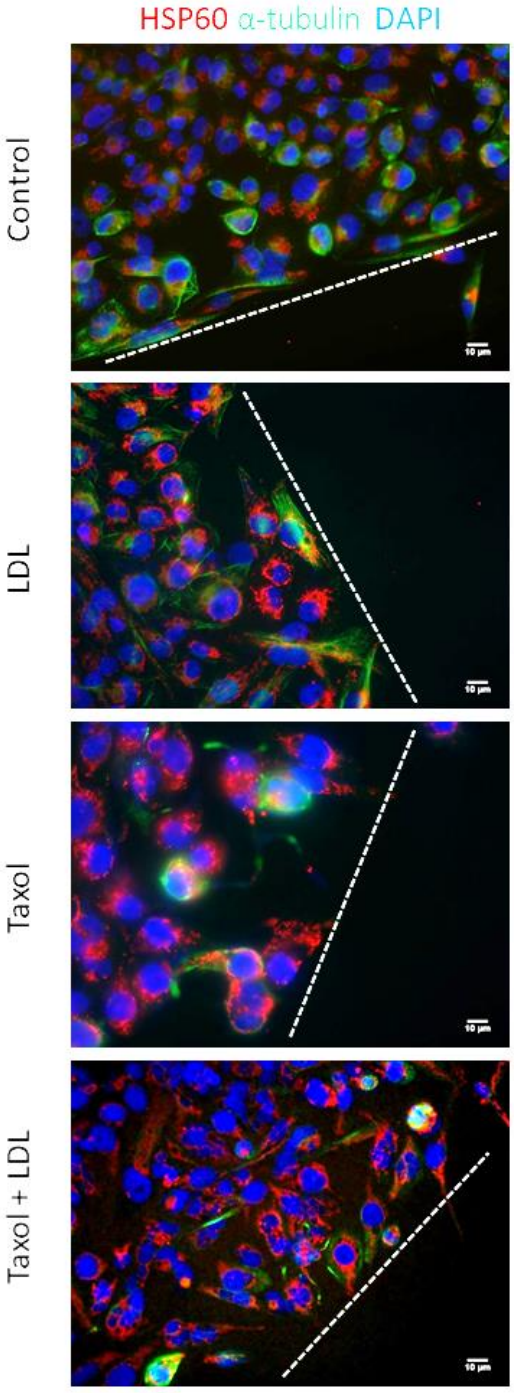
Supplementary Figure 2 Effect of LDL in mitochondrial morphometrics (size and elongation) in MCF-7 and MDA-MB-231 migrating cells.

Quantification of mitochondrial morphometrics (size and elongation) of MCF-7, control and LDL-exposed MDA-MB-231 migrating cells (n ≥ 18 cells). **a)** Mean size of mitochondria; **b)** Mean elongation of mitochondria. Data are presented as mean ± s.d. Statistical significance was determined by two-tailed Student *t*-test. **, $P < 0.01$; ***, $P < 0.001$.



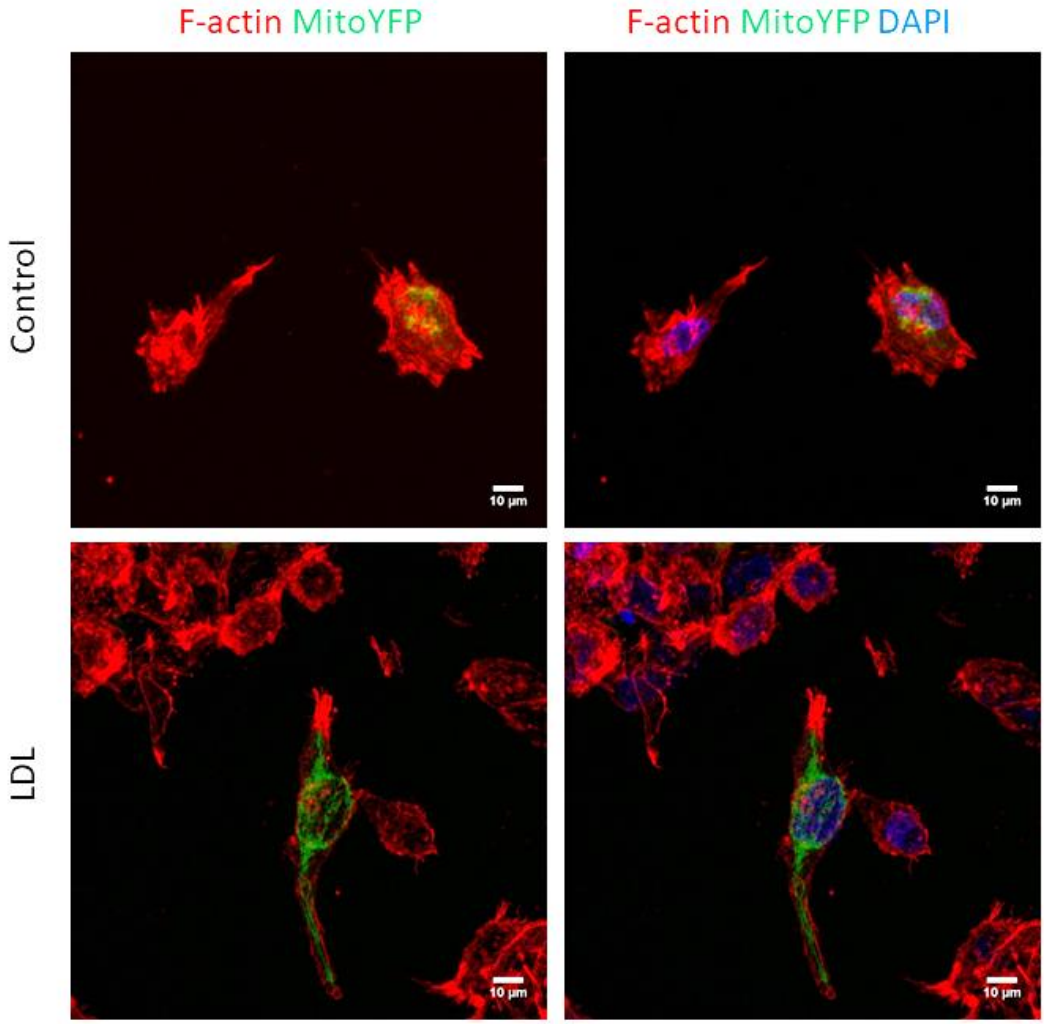
Supplementary Figure 3. Analysis of mitochondrial mass across several breast cancer cell lines (Luminal A – MCF-7; Luminal B – BT-474; Triple-Negative – MDA-MB-468 and MDA-MB-231).

a) Mitochondrial mass represented as mean fluorescence intensity (MFI) of MitoTracker Deep Red relative to the MCF-7 group (n = 5/6 from two independent experiments **b)** Relative mtDNA content based on the mitochondrial *ND1* gene relative to the nuclear *B2MG* gene relative to MCF-7 group (n= 5/6 from two independent experiments). Data are presented as mean ± s.d. Statistical significance was determined by two-tailed Student t-test. *, $P < 0.05$; ***, $P < 0.001$.



Supplementary Figure 4. Taxol-induced phenotype is restored by LDL and is dependent on mitochondria-cytoskeleton interactions.

Representative epifluorescence images of Control (upper panel), LDL (second panel), Taxol 20 nM (third panel) and Taxol 20 nM+LDL exposed MDA-MB-231 cells (lower panel). MDA-MB-231 cells are stained with α-tubulin in green, HSP60 (mitochondria) in red and DAPI (nuclei) in dark blue. The dashed white line represents the wound front edge. Images were acquired with a 40x oil objective. Scale bar 10 µm.



Supplementary Figure 5. LDL-exposed MDA-MB-231 cells present increased lamellipodia formation.

Representative confocal images of control (upper panel) and LDL-exposed (lower panel) MDA-MB-231 cells transfected with MitoYFP and stained with Phalloidin-647 (F-actin) and DAPI (nuclei). Images were acquired with a 63x oil objective. Scale bar equals 10 µm.